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STUDIES ON TYPHUS VIRUS IN THE LOUSE*

F. Breinl

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The investigations of Nicolle have shown that the louse is the only carrier of the typhus virus. As the louse infests only man and monkeys, the method of injection must be used to test for the virus in the ordinary laboratory animals—guinea-pigs and rabbits. The whole louse or the intestines are emulsified in normal salt solution for injection. The results of Rocha Lima, who discovered Rickettsia prowazeki in the epithelium of the intestines, made it probable that this organ is the seat of the typhus virus. Several authors consider Rickettsia the probable cause of typhus. Without trying to settle this difficult question, we studied the behavior of the typhus virus in experimental infection and immunization, after it had been kept for some time in the organism of the louse. These investigations were made possible by Weigl's fundamental discovery of a way to transfer the virus from one louse to another repeatedly without the necessity of infecting a mammal. Weigl [1] showed that with the help of a fine glass capillary tube affixed to a syringe it is possible to inject infectious material into the rectum of a louse. He injected the blood of typhus patients during fever or an emulsion of infected lice; 1-3 weeks after the injection changes such as Rocha Lima described can be noticed in the intestine of some of the lice; the endothelium is swollen in some places; rickettsias are developing in the cells; some endothelial cells burst and discharge the rickettsias into the intestinal tube, from whence they are evacuated with the feces. For isolating the intestines the louse is placed on a glass slide, the chitinous membrane is cut behind the third pair of legs as far as the middle without injuring the intestines, then the head and body are pulled apart with fine dissecting needles, whereupon the isolated intestines remain on the glass slide between the rest of the body. They are then triturated with normal salt solution in a small glass mortar and can be used for infecting a new series of lice. In this way the virus can be cultivated in lice passages for a long time. We had the

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* These investigations are a continuation of former work (Jour. Infect. Dis., 1923, 33, p. 60). They were started in Professor Weigl's laboratory in Lemberg together with Professor E. Weil, who contracted a fatal typhus infection during the work. After his death the experiments were continued and terminated in the Hygienic Institute of the German University in Prague.

[1] Przegl. epidemiol., Warsaw, 1920.

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opportunity of testing three different strains of typhus after divers passages, that had been cultivated in the louse, and we found that the louse virus differs from guinea-pig virus in various points.

EXPERIMENTS WITH THE LOUSE STRAIN EX. 60

Lice were fed on typhus patient 60 when the fever was at its height. Some of them discharged rickettsias 10 days later. A new series of lice was infected by Weigl's method with the emulsion of the gastro-intestinal tract of these animals—Ex. 60 first passage—and was fed for 10 days on men immune to typhus. At the end of this time the intestines of two lice of this series were triturated with 5 c c. of salt solution. Five guinea-pigs (1-5) were injected intraperitoneally with 1 c c. of this emulsion. The temperature of these animals was taken for 3 weeks beginning on the day after the injection (charts 1 and 2, thick temperature curve).

After the lapse of this time all the animals were superinfected with 0.1 of guinea-pig passage brain to ascertain the specific nature of the fever by the proof of immunity (charts 1 and 2, thin temperature curve). The thick horizontal line at 39.2 degrees shows the highest limit at which the temperature can still be considered normal.

In this experiment, the abnormally short time of latency is remarkable: In 3 animals the rise of temperature set in after 5 days, in one after 4 days, only in guinea-pig 3 the fever set in after a normal incubation of 7 days.[2] The two guinea-pigs No. 1 and 4 died during the fever (No. 1 after a premortal decline of temperature), and it was impossible to find the cause of death by pathologic or bacteriologic examination. These animals thus apparently died of typhus, an exceptionally rare occurrence when working with guinea-pig brain virus. The fever lasted far longer than usual in 2 of the surviving animals: guinea-pig 3 had fever for 13, guinea-pig 5 for 15, days. All these unusual occurrences—shorter period of latency, longer duration of fever, and death of 2 animals—are a sign that the infection was very severe in this experiment.

The infections with the 2d, 3rd and 6th passage of louse virus of the strain Ex. 60 took a similar course. The intestines of 4 lice of the 2d passage were emulsified in 5 c c. of salt solution. Guinea-pigs 6-10 were injected intraperitoneally with 1 c c. each. The intestines of 2 lice of the 3rd passage were emulsified in 5 c c. of salt solution, guinea-pigs 11-15 receiving 1 c c. intraperitoneally each. In both

[2] We call a normal period of latency the one noticed after infection with 1/20 of passage guinea-pig brain.

experiments the abnormally short time of latency was again noticeable. In some animals the rise of temperature set in after 48 hours (guinea-pigs 6, 9). The irregularity of the temperature curves is especially

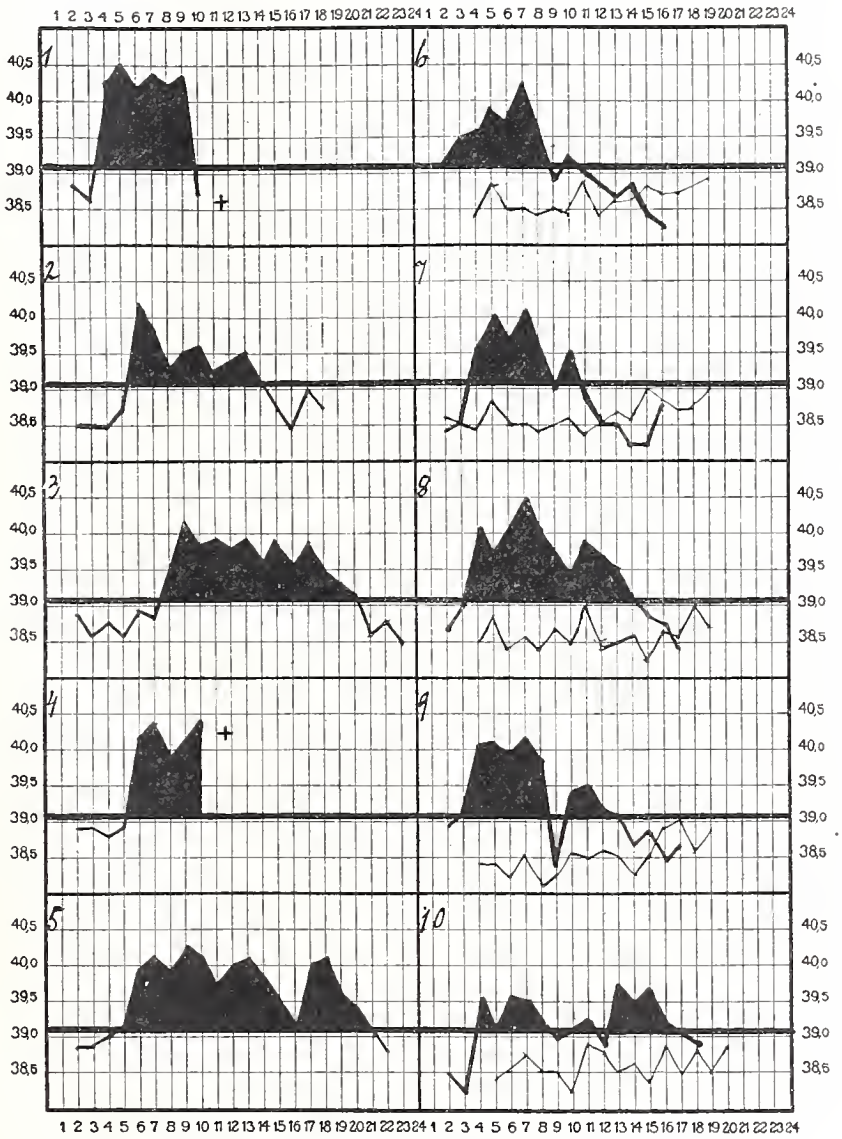

Chart 1.—Ex. 60, passage 1 and 2.

remarkable: in the majority of cases the fever lasted longer than after infection with guinea-pig virus and was often interrupted by periods of normal temperature. In one case the fever was nearly rudimentary (guinea-pig 10). We saw regularly similar curves after simultaneous

injection of virus and homologous immune serum. The negative results of the superinfection shows that the fever was really typhus in every case.

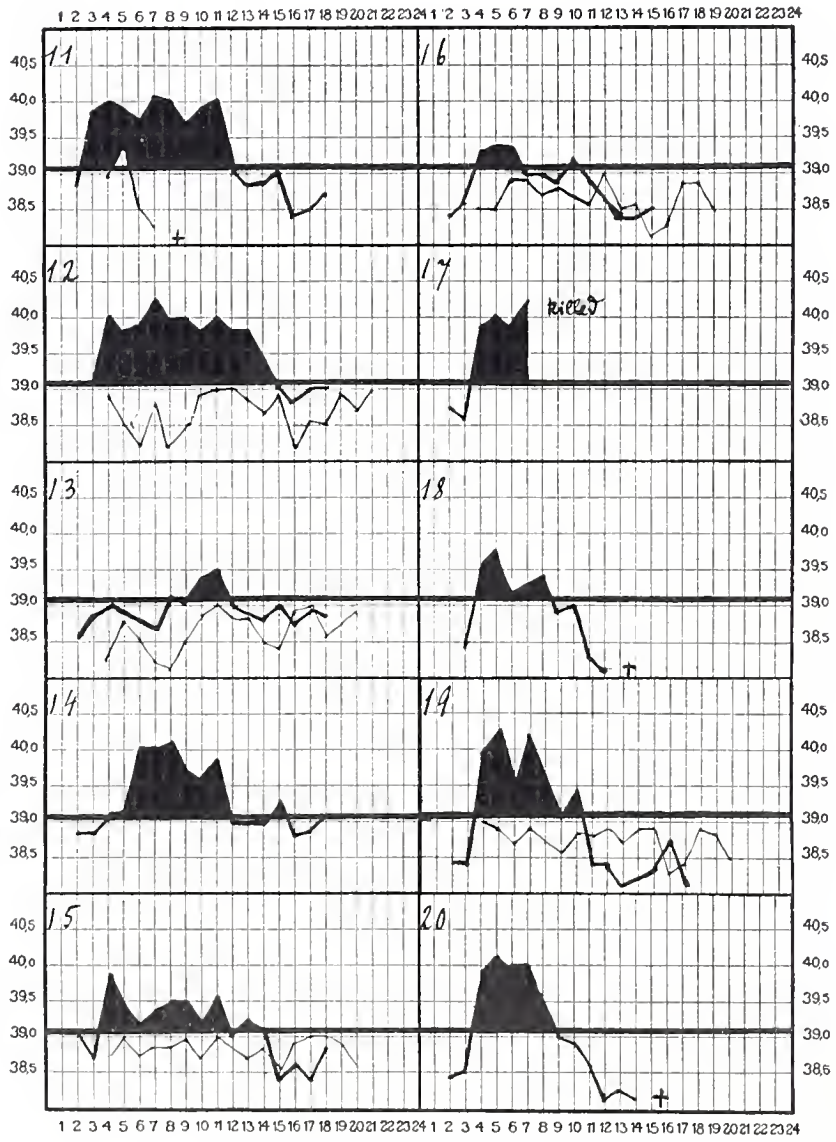

Chart 2.—Ex. 60, passage 3 and 6.

Ten louse intestines of the 6th passage were emulsified in 12 c c. of salt solution. Guinea-pigs 16-20 were injected intraperitoneally with 1 c c. each. In this series the short period of latency and the modification of the fever curve are distinctly marked. Guinea-pig 16 showed

attenuated fever lasting only 2 days; in this case the infection was almost symptomless. In the case of guinea-pig 20 the fever reached the normal height, but lasted only 4 days. Two animals (guinea-pigs

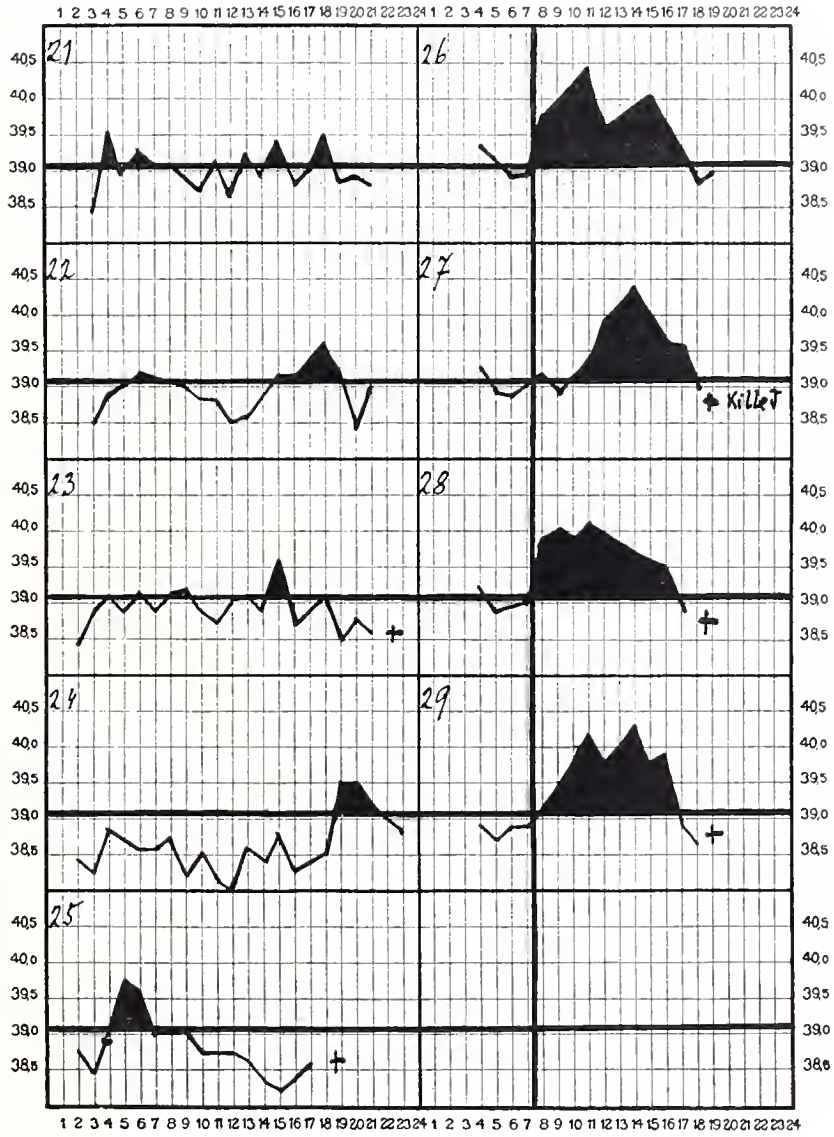

Chart 3.—Titration of louse virus. Passage from guinea-pig 17.

18 and 20) died during the experiment after a great loss in weight, without showing any other pathologic sign. Guinea-pig 17 was bled on the 4th day of fever, and 1/100 part of its brain was injected intraperitoneally into 4 guinea-pigs (chart 3, 26-29). A typical typhus

infection developed in all of them. Two animals died after the temperature had returned to normal, again without any pathologic findings. In subsequent passages no more deaths occurred.

The contents of the virus in the lice intestines were tested by experiments on rabbits. The injection of lice intestines of the 2d, 4th and 6th passage provoked unexceptionally a distinct production of agglutinin against Bacillus X 19. The 1st passage was not examined. I give only the results of the experiment with the 6th passage in extenso (table 1). Three rabbits were injected intraperitoneally with 2 c c. of the same emulsion that had been used for the guinea-pig experiment.

TABLE 1

Results of Injection of Intestines of Lice into Rabbits

Rabbits	Titer	Day of Infection		7th Day		14th Day	
		HX_{19}	OX_{19}	HX_{19}	OX_{19}	HX_{19}	OX_{19}
1	1 : 5	++	+++	+++	+++	+++	+++
	1 : 10	—	+	+++	+++	++±	+++
	1 : 20	—	—	++±	+++	++	+++
	1 : 50	..	..	±	++	+	++
	1 : 100	..	..	—	+	—	+
2	1 : 5	—	±	++	++±	+++	+++
	1 : 10	—	—	+	++	++	++±
	1 : 20	..	..	—	—	+	++
	1 : 50	..	..	..	..	—	—
3	1 : 5	—	±	+++	+++	+++	+++
	1 : 10	—	—	+++	+++	++±	+++
	1 : 20	..	..	++±	+++	++	+++
	1 : 50	..	..	+	++±	+	++±
	1 : 100	..	..	—	+	—	+

In two cases the agglutination had already reached its full height on the 7th day, which is never the case after infection with guinea-pig virus. In the 3rd animal distinct production of agglutinins was noticeable on the same day, but the quantity increased to double the amount at the end of the second week. This accelerated production of antibodies after infection with the louse virus is such a regular occurrence that it must have a definite cause. In some cases, the agglutinins diminished distinctly on the 14th day in comparison to the height reached on the 7th day. All the rabbits were superinfected with $\frac{1}{20}$ of a virulent guinea-pig brain 4 weeks after the end of the first experiment, and were examined serologically a week, and a fortnight, later; but the minimal agglutination which still existed at that time (1 : 10 +) was not influenced by the reinfection. The animals had thus acquired active immunity by infection with louse virus.

After these experiments there can be no doubt that it is possible to keep the typhus virus alive for several generations in the organism of the louse. The course of infection after injection of louse virus differs from that after injection of guinea-pig virus in the following points: (1) the period of latency is much shorter on an average; (2) the fever curve is less regular, often lower, shorter, or interrupted by periods of normal temperature; (3) the animals sometimes die of the infection. In the rabbit agglutinins appear 7 days after infection.

We will try to explain these abnormal symptoms have a common cause. Our earlier investigations cleared up the relation between the length of the period of latency and the infecting dose: the larger the chosen infecting dose of virus, the shorter the period of latency. But we were not able to obtain regularly a rise of temperature earlier than 6 days after infection, even when using the highest doses of virus. The greatest quantity of virus used in these experiments was 100,000 infecting doses; it was therefore thought possible that the unusually short period of latency had been evoked by quantities of virus far exceeding the dose mentioned. To measure the contents of the virus of the artificially infected louse, different quantities of the intestines were tested. The intestines of a louse were emulsified in 10 c c. salt solution, and diluted 1 : 100 and 1 : 1000. Guinea-pigs 21 and 25 (chart 3) were injected with 1 c c. of the dilution 1 : 100. The fever reaction was low in all cases, but the following reinfection showed that immunity had been obtained: Five animals injected with $\frac{1}{1,000}$ of louse intestines had normal temperatures during 3 weeks and were susceptible to a reinfection with guinea-pig virus: the quantity of virus contained in $\frac{1}{1,000}$ of louse intestines is too small to infect a guinea-pig. The examined intestines thus contained 100 infectious doses of virus. This experiment shows that the precipitate rise of temperature cannot be explained by an abnormally great quantity of virus, as all the animals so far described received less than 100 doses.

If a guinea-pig is injected with $\frac{1}{100,000}$ of a brain containing virus, which is one infectious dose, as is $\frac{1}{100}$ of louse intestines, it reacts with a rise of temperature of normal length and height after a remarkedly lengthened period of latency. But curves such as those shown by guinea-pigs 21 and 25 (chart 3) are never observed. Similar curves, however, appear regularly after a simultaneous injection of virus and immune serum. We must therefore presuppose that here too an immunizing substance besides the virus is at work. This immunizing substance can be only dead typhus virus. Although

we had never been able to produce immunity in guinea-pigs or rabbits by previous treatment with large quantities of dead guinea-pig brain virus, it was possible that the louse virus would act differently in this respect. The intestines of 80 lice (6th passage) were triturated in 8 c c. of salt solution and 0.5% of phenol was added. The emulsion stood at room temperature for 20 days; then 4 rabbits were injected intravenously with 2 c c. each (table 2).

All the animals showed marked production of agglutinin 7 days later, which began to decrease in 3 animals after a fortnight (table 4). Reinfection showed that all the animals had become actively immune by this one injection. This experiment proves that the intestines of the artificially infected louse contain dead typhus virus. We must

TABLE 2

Immunization of Rabbits by Injection of Louse Virus

Rabbits	Titer	Day of Infection		7th Day		14th Day	
		HX_{19}	OX_{19}	HX_{19}	OX_{19}	HX_{19}	OX_{19}
4	1 : 5	—	—	+++	+++	+++	+++
	1 : 10	—	—	+++	+++	+++	+++
	1 : 20	..	..	++±	+++	+++	+++
	1 : 50	..	..	+	+	++±	+++
	1 : 100	..	..	—	—	++	++
5	1 : 5	±	+	+++	+++	+	++
	1 : 10	—	—	+++	+++	+	+
	1 : 20	..	..	++±	+++	—	±
	1 : 50	..	..	+	++	—	—

suppose that the quantity present far exceeds 100,000 doses, for we were not able to produce immunity with the same quantity of dead guinea-pig virus. The antigen power of the dead louse virus could be shown in the guinea-pig experiment too. Nine guinea-pigs were injected subcutaneously with the emulsion of the intestines of 25 artificially infected lice after an interval of 5 days the same injection was made. Six days later they were infected with 0.05 virulent guinea-pig brain, 30-38 (chart 4). In three cases the infection was absolutely symptomless; the other animals had only low fever after a period of latency 3-4 days longer than normal. The reinfection 4 weeks later proved all the animals to be immune. Probably all the guinea-pigs could have been protected completely by a stronger immunization. The mechanism of the immunity must be cleared up by further examinations. Rocha Lima has already previously obtained similar findings.[3] These

[3] München. med. Wchnschr., 1918, 65, p. 1454.

experiments show that by injecting an emulsion of intestines of infected lice, dead virus is incorporated besides the living virus. The infected louse contains a great quantity of virus, of which the greater part is

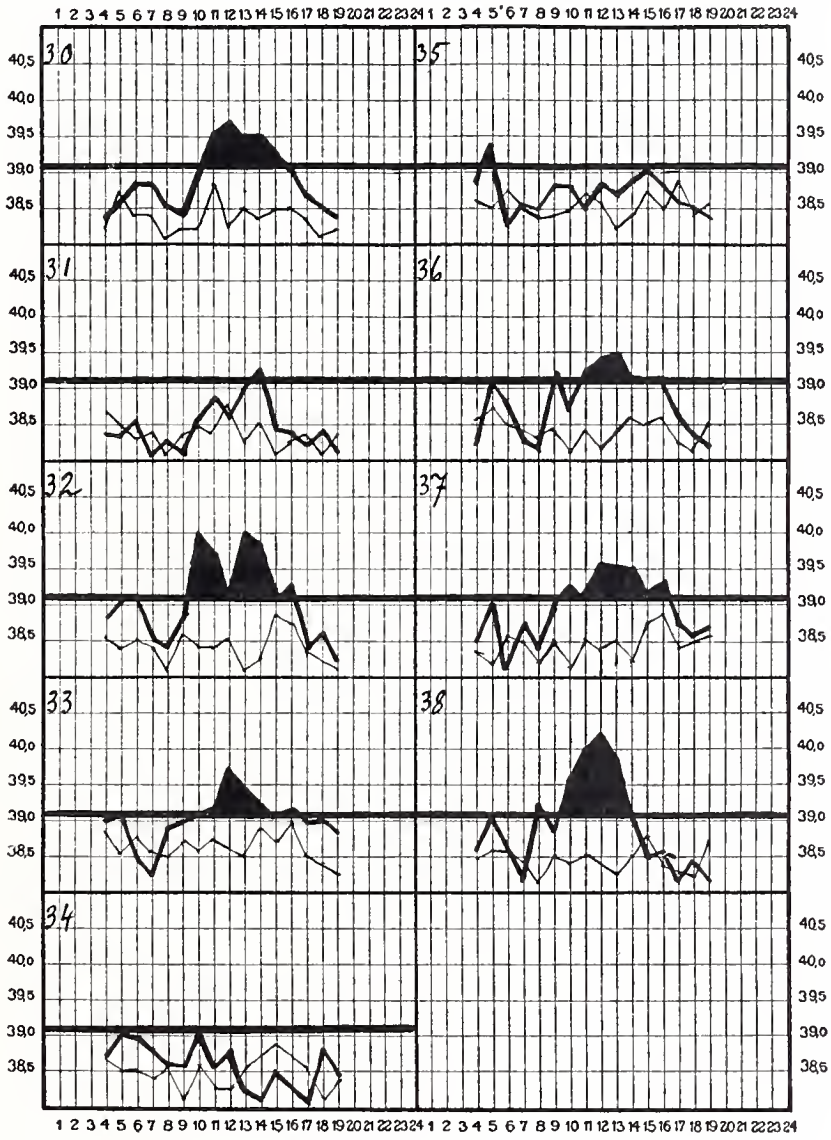

Chart 4.—Immunization by louse intestines.

dead. It is well known that dead germs or their metabolic products often precipitate infection when they are injected simultaneously with the homologous living bacteria into the organism of a susceptible animal. Certain metabolic matter produced by highly infectious parasites (Bail's

aggressins) particularly accelerates and aggravates the infection. These products have also a high immunizing power. As the typhus virus multiplies rapidly in the louse, the production of similar metabolic matter is probable. The dead virus itself or metabolic products of the virus are the cause of the abnormally short period of latency. On the other hand, the rudimentary, shortened or interrupted fever, as a sign of beginning immunity, can be explained by the effect of the dead virus.

In a previous article we were able to show that the fever reaction after simultaneous injection of virus and immune serum is smaller the larger the quantity of serum in proportion to the infecting virus. In the same way, the immunizing action of the dead virus preponderated in guinea-pigs 21-25 which had been infected with a small dose of virus, whereas in the other experiments in which a larger infecting dose had been used, the quality of precipitating infection generally outweighed the immunizing action; in consequence of the differences in the natural resistance of divers animals, various intermediate forms appeared. The death of some of the animals was evidently caused by the rapid increase of the virus and the following extensive destruction of tissue. The dead virus probably had a primary toxic effect, for several animals died, even after very low fever. Nearly half of the rabbits injected with louse virus died within 8 weeks without any cause determinable by anatomic or bacteriologic investigations.

Similar results were obtained with 2 other typhus strains, Ex. 61 and Ex. 66. Further investigations were made to determine whether the virus remains localized in the gastro-intestinal tract when a louse has been infected artificially, or whether it is found in the other organs also. For this purpose the intestines were dissected as completely as possible and with the utmost care so as to avoid contamination of the other organs by feces. Twenty lice of the strain Ex. 61, third passage, were dissected in this way, the intestines being triturated with 24 c c. of salt solution, and the remainder of the bodies with 10 c c. of salt solution. Rabbits 6 and 7 were each injected intravenously with 2 c c. of the intestinal emulsion and rabbits 8 and 9 with 3 c c. of the emulsion of body residue. Whereas the emulsion of intestines provoked a great production of agglutinins in 2 animals (6 and 7), the treatment with the emulsion showed only a slight increase of the antibodies normally present in rabbit 8, and an only just perceptible agglutination at 1 : 5 in rabbit 9 (table 3). This minimal production of antibodies is probably caused by small quantities of virus adhering to the body residue. The question of the localization of the virus in the

louse is of great importance for understanding the natural typhus infection in man. If the man is infected by the bite of a louse, this could only be by the virus contained in the louse saliva, which flows into the wound while the insect is sucking. The intestine-free body which contains salivary glands has, as we have shown, little if any virus. It seems therefore that the infection is caused by the contact with lice intestines—by embrocating crushed lice or their feces into the skin.

The guinea-pig experiments reported in our former paper have shown that the virus can be found in the organs and in the blood some time after the temperature has returned to normal. This question has not been investigated systematically with human material.

TABLE 3

RESULTS OF EXPERIMENTS WITH TYPHUS STRAINS EX. 61 AND EX. 66

Rabbits	Titer	Day of Infection		7th Day		14th Day	
		HX_{19}	OX_{19}	HX_{19}	OX_{19}	HX_{19}	OX_{19}
6	1 : 5	—	—	+++	+++	+++	+++
	1 : 10	—	—	++±	+++	+++	+++
	1 : 20	..	..	++	+++	++	+++
	1 : 50	..	..	±	++	±	++
	1 : 100	..	..	—	±	—	+
7	1 : 5	—	—	++±	+++	+++	+++
	1 : 10	—	—	++±	+++	++±	+++
	1 : 20	..	..	++±	+++	+±	+++
	1 : 50	..	..	±	++±	±	++
	1 : 100	..	..	—	+	—	+
8	1 : 5	++	++	++	++	++	++±
	1 : 10	±	+	+	++	+	++
	1 : 20	—	—	—	—	±	+
9	1 : 5	—	—	—	—	+	+
	1 : 10	—	—	—	—	—	—

Three lice were fed for several hours on a patient the first day of apyrexia, and were then kept for 7 days on men immune to typhus. Their intestinal emulsion provoked abortive fever in 3 animals—twice after a short period of latency—and in 1 animal a symptomless infection; all the animals acquired active immunity. This proves that the virus is present in human blood for at least 24 hours after the temperature is normal. Rocha Lima obtained similar results in 1 of 6 experiments.[4]

A louse virus strain was obtained by anal infection of lice with virulent guinea-pig brain emulsified in salt solution. Two weeks later their intestines were injected into the peritoneal cavity of guinea-pigs.

[4] München. med. Wchnschr., 1916, 63, p. 1381.

All the animals had fever lasting for 7-9 days after an incubation of 3-4 days. One guinea-pig was bled when the fever was at its height, and its brain was used for injecting guinea-pigs. It was once more evident that the virus had undergone no change by passing through lice.

SUMMARY

With Weigl's method of injection it is possible to pass the typhus virus from louse to louse repeatedly.

The course of the disease after injection of emulsified louse intestines differs from that after injection with virulent guinea-pig brain in these points: (*a*) In the guinea-pig the period of latency is generally much shorter. Often the fever is abridged or interrupted by periods of normal temperature, and the height of the fever curve is less than after infection with guinea-pig virus. Some animals die of the infection. (*b*) The agglutinin for the bacillus X 19 appears much sooner in the rabbit after injection of louse virus. As a rule, the half of the maximal titer is reached on the 7th day.

Production of aggluitnin and active immunity can also be obtained in the rabbit by injecting emulsified louse intestines, to which phenol has been added. Guinea-pigs can be immunized also by repeated treatment with dead louse virus.

The peculiarities of the infection following the injection of the louse virus are explained by the co-existence of dead and living virus.

The typhus virus multiplies considerably within the louse, but does not become more virulent.

Ten days after infection the intestines of a louse contain 100 doses of living virus.

The body of the louse contains only small quantities of virus after the intestines have been removed. From this fact we can draw conclusions as to the way of natural infection in man.

Lice fed on a patient one day after the fever had disappeared, proved to be infectious 7 days later.

It is possible to infect lice with an emulsion of virulent guinea-pig brain.

ACTION OF TYPHOID BACILLUS ON RUSSELL'S DOUBLE-SUGAR MEDIUM

George W. Wheeler

From the Division of Laboratories, New York Hospital

Russell's double-sugar medium was first described in 1911.[1] This medium is a meat-extract agar, containing 0.1% of dextrose, 1.0% of lactose, and an indicator. It is used in the form of an agar slant, with a long butt, the slant surface and the butt being about equal in length. The medium is inoculated by making a deep stab into the butt, then a streak along the slant surface.

Russell's medium has found a wide application in the preliminary differentiation of members of the typhoid-colon group. When a colony of an organism belonging in this group is transferred to double-sugar medium, the following changes may be observed after 24 hours: (a) If the organism ferments dextrose and lactose with acid and gas, slant and butt are acid, and there are gas bubbles throughout the medium (for example, Bact. coli). (b) If the organism ferments dextrose with acid and gas and does not ferment lactose, the slant is alkaline, the butt is acid, and there are gas bubbles chiefly in the butt (for example, paratyphoids—intermediates). (c) If the organism ferments dextrose with acid, but no gas, and does not ferment lactose, the slant is alkaline, the butt is acid, and there are no gas bubbles (for example, typhoid—dysentery). (d) If the organism ferments neither dextrose nor lactose, the slant and butt are alkaline (for example, Bacillus alkaligenes).

It is possible, therefore, by means of this medium, to determine the subgroup in which an organism belongs; its complete identification is thereby facilitated.

More recently, Krumwiede and Kohn[2] have advocated the addition of 1.0% of saccharose to Russell's medium for the purpose of detecting certain members of the intermediate group which ferment dextrose and saccharose with acid and gas, but which ferment lactose slowly or not at all. On Russell's medium these organisms might be mistaken for members of the paratyphoid group.

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[1] Jour. Med. Res., 1911, 25, p. 227.
[2] Jour. Med. Res., 1917, 37, p. 225.

The alkaline slant and acid butt observed, after 24 hours, in a double-sugar tube when inoculated with the typhoid bacillus, were explained by Russell, in his original communication, as follows: "The entire point of the medium rests upon the difference in changes produced by the growth of the typhoid bacillus under aerobic and under the imperfect anaerobic conditions found in the butt of the tube, where the bacillus obtains its oxygen by breaking down the glucose with the liberation of considerable acid; on the surface, however, in the presence of free oxygen, no acid is formed."

Nichols [3] questions the accuracy of the foregoing explanation on the grounds that it does not take into account the fact that the typhoid bacillus ferments glucose with acid end products under both aerobic and anaerobic conditions. This author considers that fermentation of glucose takes place on the slant as well as in the butt, although direct experimental evidence of the fermentation of 0.1% of glucose is not offered, and that the small amount of acid formed from the 0.1% of glucose might volatilize and leave the medium apparently unaffected. In support of this hypothesis, evidence is presented to show that the typhoid bacillus, growing on Russell's medium, does produce considerable quantities of volatile acid and that this acid is largely, if not entirely, CO_2. The neutral or alkaline reaction on the slant is attributed to the escape and neutralization of these acid products of fermentation.

In his summary the author states that: "CO_2 is produced by the typhoid bacillus in significant amounts both from sugars and from proteins. The appearance of the Russell double-sugar tube during the growth of the typhoid bacillus is not due to direct oxygen requirements. It is due (a) to the retention of CO_2 in the butt of the tube and its escape from the slant, and (b) to alkaline reversion of other acids."

In a second communication, Nichols and Wood [4] report the findings of several other observers as indicating that some of the CO_2 produced by the typhoid bacillus may be respiratory. Their conception of the importance of CO_2 production as a factor in causing the changes seen in a typhoid Russell tube is expressed as follows: "The process, whatever it is, is one in which CO_2 is produced on the slant. It escapes, leaving an accumulation of nonvolatile alkaline products. In the butt the same process occurs, but the CO_2 is retained by the medium and makes it acid. By degrees, however, the alkali from the slant diffuses downward and renders the whole tube alkaline."

[3] Jour. Infect. Dis., 1921, 29, p. 82.
[4] Ibid., 1922, 30, p. 320.

In their summary the authors state that: "The CO_2 produced by the typhoid bacillus in the Russell double-sugar tube is probably fermentative as well as respiratory. Phenol red is the best indicator as it shows both the acid change in the butt and the alkaline change in the slant. The slant is not unchanged. It becomes alkaline and gradually the alkalies spread down and turn the butt alkaline."

There are, therefore, two different explanations for the alkaline slant and acid butt seen in a typhoid Russell tube after 24 hours' incubation. According to Russell, the oxygen supply is the determining factor. On the slant, where oxygen is abundant, dextrose is not fermented and the slant remains alkaline; in the butt, where oxygen is scanty, the dextrose is fermented and the butt becomes acid. According to Nichols, and to Nichols and Wood, the changes observed are not due to direct oxygen requirements. The dextrose is fermented in the slant as well as in the butt, and CO_2 is produced. The ultimate disposal of this CO_2 is the determining factor. It escapes from the slant surface, and the residual alkaline products render the slant alkaline; it is retained in the butt, and the butt becomes acid. Eventually the alkali in the slant diffuses downward, and the butt becomes alkaline.

Our experiments with Russell's medium, while confirming some of the above observations, have led to conclusions differing somewhat from both of the views expressed.

TECHNICAL

The medium employed was a meat-extract agar containing 0.3% of Liebig's meat-extract, 1.0% of Bacto-peptone,* 0.5% of NaCl (C. P.), and 1.5% of Bacto-agar,* in distilled water. The reaction was adjusted to P_H 7.3—7.4. This stock medium was bottled in 200 c c. amounts, sterilized in the autoclave for 20 minutes at 15 lbs. pressure, and stored in the icebox. In preparing Russell tubes, bottles of the stock medium were liquefied in a boiling water bath, then 10 c c. of a solution containing 20% of lactose and 2% of dextrose, and 2 c c. of Andrade indicator or 5 c c. of 0.04% aqueous solution of phenol red were added to each bottle. The medium was tubed in 10 c c. amounts, sterilized in the Arnold sterilizer for 15 minutes on each of 3 consecutive days, and stored in the icebox without slanting. Just before inoculation, the required number of tubes was placed in boiling water bath for 15 minutes, then slanted in the icebox in such a way as to give a slant and butt of about equal length.

* Prepared by The Digestive Ferments Co., Detroit, Mich.

In many of the experiments 4 different tubes were used; Russell's medium, with Andrade indicator or phenol red, and the same stock medium, with no sugars added, but containing Andrade indicator or phenol red. Tubes of this stock medium to which no sugars were added showed no acid and no gas when inoculated with Bac. coli; it may, therefore, be considered as sugar-free.

Whenever two tubes were coupled together, the connection was made by means of a U-tube having the same diameter as the culture tubes and held in place by short lengths of thick-walled rubber tubing. This device was described by Nichols and Schmitter.[5] All joints, rubber tubing, and rubber stoppers were covered completely with one or more coats of thick shellac.

Inoculations of slant and butt were made with a single loopful of a 24-hour broth culture of a stock strain of Bac. typhosus. All cultures were incubated at 37 C.

EXPERIMENTAL

A. Experiments were performed to determine (a), whether or not all of the dextrose is fermented in the slant and in the butt of a typhoid Russell tube during the first 24 hours' incubation, and, if so, the number of hours required for the completion of the process; and (b) whether or not fermentation is complete when more than 0.1% of dextrose is present.

Exper. 1.—Four c.c. of Russell's medium were placed in each of 12 culture tubes and slanted. These tubes had a slant surface of about the same dimensions as a standard Russell tube, but had no butt. The 12 slants and 12 standard tubes (slant and butt) were inoculated with typhoid bacilli. At 3-hour intervals one slant and one standard culture were placed in boiling water bath for 5 minutes, sterile N/10 NaOH was added to restore the original reaction of the medium; the tubes were cooled to 45 C., inoculated with 1 loopful of a 24-hour broth culture of paratyphoid B., and allowed to solidify without slanting. After 24 hours these paratyphoid B cultures were examined for acid and gas. Absence of acid and gas was considered as evidence that there was no demonstrable amount of dextrose present at the time the paratyphoid inoculations were made.

Results: The slants that were liquefied and reinoculated after 3, 6, and 9 hours showed acid and gas in diminishing amounts with paratyphoid B.; after 12 hours slight acidity, no gas; after 15 hours, 18 hours, etc., no acid, no gas. The standard tubes that were liquefied and reinoculated after 3, 6, 9, and 12 hours showed acid and gas in diminishing amounts with paratyphoid B; after 15 and 18 hours, slight acidity, no gas; after 21 hours, 24 hours, etc., no acid, no gas.

Exper. 2.—Standard tubes of double-sugar medium containing 0.1%, 0.2%, etc., up to 1.0% of dextrose were inoculated, slant and butt. After 24 hours the

[5] Jour. Med. Res., 1906, 15, p. 113.

tubes were liquefied, readjusted, and reinoculated with paratyphoid B, as in the preceding experiment. Twenty-four hours after reinoculation the 0.1% tube showed no acid, no gas; 0.2% tube, slight acidity, few gas bubbles; all other tubes marked acidity and gas production. In a similar experiment the typhoid Russell cultures were connected, at the time of inoculation, with tubes containing alkaline pyrogallol. After 24 hours the culture tubes were disconnected, liquefied, readjusted, and reinoculated with paratyphoid B. In this series the paratyphoid B. produced neither acid nor gas in the 0.1% tube. Acid and gas formation were, however, more marked in the 0.2%, 0.3%, and 0.4% tubes than in the corresponding tubes in the aerobic series.

Conclusions.—All of the dextrose in the slant of a typhoid Russell tube is fermented within 15 hours; in the butt, within 21 hours. If 0.2%, or more, of dextrose be present it is not fermented completely within 24 hours. The results in Exper. 2 suggest that fermentation of dextrose takes place more promptly under aerobic than under anaerobic conditions.

B. Experiments were also performed to determine the effects produced in a typhoid Russell tube when it is incubated under relatively strict anaerobic conditions. According to Russell's explanation, slant and butt should be acid. If Nichols' idea is correct, slant and butt should be acid if volatile acids are retained within the tube, but the slant should be alkaline if volatile acids escape from the tube.

EXPER. 3.—Russell and sugar-free tubes, inoculated with typhoid bacilli, were connected with a water suction pump and mercury gage, by means of a piece of glass tubing which passed through a perforated rubber stopper in the mouth of the culture tube. In one set of tubes the glass tubing was sealed in a flame when the mercury registered 4 mm.; the second set was sealed when the mercury registered 180 mm.; the third set was sealed without connecting the tubes with the suction pump (760 mm.).

All of the Russell tubes became acid, slant and butt, and remained acid for 3 weeks. The plain Andrade tubes remained neutral. The plain phenol red tubes showed no change at 4 mm.; at 180 mm. the slant showed no change, the butt was slightly acid; at 760 mm. the slant was faintly alkaline, the butt slightly acid.

EXPER. 4.—The inoculated tubes were provided with perforated rubber stoppers containing an inlet tube which terminated at the bottom of the stopper and an outlet tube which extended almost to the bottom of the slant surface. Hydrogen gas, generated with Zn and H_2SO_4 and passed through alkaline pyrogallol by means of a glass wool diffusor, was allowed to flow through the tubes for one hour. Inlet and outlet tubes were sealed in flame. After 18 hours both Russell tubes showed a marked acid reaction which persisted for 3 weeks. Sugar-free tubes showed no change in reaction.

EXPER. 5.—Inoculated tubes were connected with tubes containing about 3 gms. of pyrogallol dissolved in 15 c c. of 5% KOH solution, the alkali being added just before the connection was made. After 18 hours' incubation the Russell tubes showed a marked acid reaction in the slant and the butt, which persisted for 3 weeks. The plain Andrade tube remained colorless; the plain phenol red tube showed slight acidity in the butt after 24 hours, which persisted.

Exper. 6.—A horizontal tube, 30 cm. long, 1.6 cm. in diameter, with both ends turned up at an angle of 45 degrees, was partially filled with a solution of alkaline pyrogallol. At one end a typhoid Russell tube was attached; at the other end, an uninoculated tube of the same medium. There was a free passage for gases from the inoculated to the uninoculated tube, over the surface of the pyrogallol in the horizontal tube. After 24 hours' incubation the inoculated tube showed an acid slant and butt which persisted for 3 weeks. The uninoculated tube showed no change in reaction.

In the control experiment, sterile water was placed in the horizontal tube, instead of alkaline pyrogallol. After 24 hours the inoculated tube showed an alkaline slant and an acid butt, while the uninoculated tube showed an acid slant with some acidity in the butt. After 4 days the uninoculated tube was uniformly acid throughout.

This experiment was modified by placing a watery solution of the indicators, Andrade or phenol red, in the horizontal tubes, with an inoculated tube at one end and a tube of alkaline pyrogallol at the other. The same results were observed—marked acidity in the inoculated tube, no change in the indicator solutions. In the controls, using sterile water instead of alkaline pyrogallol, the indicator solutions in the horizontal tubes became strongly acid.

It may be assumed, therefore, that if any volatile acids were formed under the conditions of this experiment they were completely neutralized by the alkaline pyrogallol solution, yet the inoculated tubes became and remained strongly acid.

Exper. 7.—When a typhoid Russell tube is connected with an uninoculated Russell tube the slant of the uninoculated tube becomes acid (Nichols;[3] control tubes in exper. 6).

A typhoid Andrade Russell tube was connected with an uninoculated Andrade Russell tube and incubated for 2 days. The slant of the uninoculated tube became acid. The tubes were disconnected. The uninoculated tube was closed with a rubber stopper and placed in the water bath at 60 C. for 1 hour. The stopper was then removed and the tube was placed in the incubator. Within 4 hours the volatile acids diffused out, and the slant was neutral in reaction.

A typhoid Andrade Russell tube was connected with a tube containing alkaline pyrogallol and incubated for 2 days. The slant and butt of the inoculated tube became acid. Tubes were disconnected. The inoculated tube was closed with a rubber stopper and placed in the water bath at 60 C. for 1 hour. The rubber stopper was removed, and the tube was placed in incubator. Subcultures made from the slant at this time showed no growth. The slant and butt of this tube remained acid for 3 weeks, although there was ample opportunity for escape of volatile acids for the medium. The acidity in this tube was evidently of a different kind from that in the preceding tube.

A typhoid Andrade Russell tube was connected with alkaline pyrogallol, incubated for 2 days, then disconnected and placed in the incubator, without heating. Subcultures showed that the organisms were still living. After 7 hours, the slant and butt were still acid; after 18 hours, the slant had lost its acidity; after 6 days, the entire tube was alkaline.

Conclusions.—The results obtained in Exper. 3 and 4 show that when the oxygen supply is restricted, both slant and butt become acid. In these two instances the acid slant may be due either to deficiency of

oxygen (Russell), or to retention of volatile acids within the tube (Nichols). In Exper. 5 the results are at variance with those reported by Nichols [3] in a similar experiment. He states that: "When a double-sugar tube inoculated with the typhoid bacillus (Rawlings), is coupled up in an anaerobic system of pyrogallic acid and KOH, the appearance of the slant is the same as usual. In other words, in an atmosphere free of oxygen, no acid is apparently produced, as should be the case according to the original explanation. If a volatile acid is assumed, however, the neutral slant can readily be explained by absorption of the acid by the KOH." We are unable to find a reason for Nichols' observation of an alkaline slant when the tube is incubated in connection with alkaline pyrogallol. We have repeated this experiment many times, always with the results indicated above—marked and persistent acidity in slant and butt.

Exper. 6 seems to rule out the possibility of volatile acids being the cause of the acid slant since the results indicate either that no volatile acids were formed or that, if formed, they were neutralized by the alkaline pyrogallol. Exper. 7 was introduced for the purpose of illustrating the difference between a slant rendered acid by volatile acids alone, and one in which the acidity was due to the products of dextrose fermentation. Volatile acids diffused out of the slant within a few hours, leaving it neutral. The acids due to dextrose fermentation did not diffuse out of the slant; it remained permanently acid unless a change in reaction was brought about by subsequent bacterial growth.

C. Experiments were performed to determine the effects of volatile acids on the slant of a typhoid Russell tube when a fairly adequate supply of oxygen is provided, the volatile acids being retained within the tube.

EXPER. 8.—A typhoid Andrade Russell tube, provided with a perforated rubber stopper, was connected with a mercury manometer. Air was pumped into the tube until the level of the mercury was raised to 320 mm. The inlet tube was then sealed. The level of mercury showed no change during the first 3 hours, indicating that there were probably no leaks in the apparatus. The tube was incubated in the water bath at 37 C. with a standard, cotton-plugged, typhoid Russell tube as a control. The appearance of the tubes and the changes in the level of the mercury are recorded in table 1.

In this experiment it was assumed that the time at which the mercury level became stationary at 250 mm. represented the time at which the organisms had consumed all of the oxygen they were capable of utilizing, and the gas pressure within the closed system had reached a state of equilibrium.

During the first two days, while there was free oxygen available in the closed tube, this tube presented the same appearance as the control, although

there was no opportunity for escape of volatile acids. The subsequent changes in this tube may be explained as follows: There was very little oxygen available after the 3rd day and none after the 5th day. There was, however, a considerable accumulation of volatile acids in the tube. Due to the lack of oxygen, the absence of dextrose (exper. 1 and 2), and also, to some extent, to the presence of toxic products of metabolism, the organisms ceased to grow. When cessation of growth occurred the volatile acids confined in the closed system diffused into the medium and rendered it slightly acid throughout. This probably took place during the 5th and 6th days. A similar diffusion of volatile acids into the medium is seen when a sugar-free agar culture of typhoid bacilli is sealed with a rubber stopper.

In a control experiment a tube of alkaline pyrogallol, instead of a typhoid Russell tube, was attached to the mercury manometer and air was pumped in

TABLE 1

Appearance of Tubes and Change in Level of Mercury

Time of Growth	Mercury Level, Mm.	Russell Tube (Manometer)		Russell Tube (Control)	
		Slant	Butt	Slant	Butt
0 hours	320	neutral	neutral	neutral	neutral
16 hours	278	alkaline	strongly acid	alkaline	strongly acid
24 hours	269	alkaline	strongly acid	alkaline	strongly acid
2 days	260	alkaline	½ alkaline ½ acid	alkaline	½ alkaline ½ acid
3 days	260	alkaline	¼ alkaline ¾ ft acid	alkaline	⅔ alkaline ⅓ ft acid
4 days	258	alkaline	faintly acid	alkaline	¾ alkaline ¼ faintly acid
5 days	255	faintly acid	faintly acid	alkaline	⅘ alkaline ⅕ faintly acid
6 days	250	faintly acid	faintly acid	alkaline	alkaline
10 days	250	faintly acid	faintly acid	alkaline	alkaline

until mercury level was raised to 320 mm. On the third day the mercury had fallen to 215 mm., where it remained stationary. The difference between the drop from 320 mm. to 250 mm. with the typhoid Russell tube and from 320 mm. to 215 mm. with the alkaline pyrogallol tube may be explained by assuming that the typhoid bacillus utilized O_2 and gave back a certain amount of CO_2, while the alkaline pyrogallol took up O_2 and gave back nothing.

Conclusion.—The results observed here seem to indicate that volatile acids, even if not permitted to escape, are incapable of causing an acid reaction in the slant of a typhoid Russell tube when free oxygen is present.

D. To determine the relationship between volatile acid production and (a) the amount of oxygen present (b), the amount of dextrose in the medium. In these experiments the culture tubes were connected

with other tubes containing various indicators. The amount of oxygen present in the different tubes is expressed, numerically, in terms of c c. of atmospheric air included within the tubes when they were sealed. These estimations were made at the end of each experiment by filling the culture tubes, connecting tubes, and indicator tubes with water from a buret and recording the amount of water required. In those instances in which a partial vacuum was created within the tubes, the reduction in pressure is expressed in terms of mm. of mercury, as indicated by a manometer gage connected with the suction pump.

EXPER. 9.—An inoculated and an uninoculated Russell tube with perforated rubber stoppers, were connected by means of glass tubing, so that hydrogen gas could be passed through both tubes. The inlet tubes terminated at the bottom of the stoppers; the outlet tubes extended nearly to the bottom of the slant surfaces. Hydrogen gas, generated with Zn and H_2SO_4, and passed through a solution of alkaline pyrogallol by means of a glass-wool diffusor, was allowed to flow through the tubes for 1 hour. The distal inlet and outlet tubes were then sealed, leaving a free passage for gases between the inoculated and the uninoculated tubes. After 24 hours' incubation, the inoculated tubes showed marked acidity of slant and butt, which persisted for 2 weeks. During this time the uninoculated tubes showed no change in reaction.

In the control experiment the two tubes were connected and sealed, as above, without replacement of air by hydrogen. After 24 hours the inoculated tube showed an alkaline slant and an acid butt; the uninoculated tube showed an acid slant and a neutral butt. After 5 days the upper half of the butt of the inoculated tube had become alkaline, the lower half remaining acid, while the uninoculated tube was acid throughout.

At the end of this experiment the inoculated tubes were liquefied, readjusted, and reinoculated with paratyphoid B, as in exper. 1 and 2. This organism produced no acid and no gas, an indication that all of the dextrose had been fermented by the typhoid bacillus. Under relatively strict anaerobic conditions, therefore, the typhoid bacillus, although it ferments 0.1% of dextrose completely, does not produce a sufficient quantity of volatile acid to show an acid reaction with Andrade's indicator or phenol red.

EXPER. 10.—This experiment was performed to show the influence of different quantities of oxygen, the amount of dextrose being constant.

Six typhoid Russell tubes were connected with tubes containing indicators, as follows:

Tube 1: Connected with a tube contaning 10 c c. of sterile water and 0.8 c c. of 0.02% aqueous solution of methyl red.

Tube 2: Connected with a tube containing 10 c c. of sterile water and 0.8 c c. of 0.02% solution of brom cresol purple. (Two different indicators were employed because it could not be determined beforehand whether the final reaction would be within the range of brom cresol purple or of methyl red).

Tube 3: Same as tube 1.

Tube 4: Same as tube 2.

The 4 indicator tubes had tubulures at the side for connection with the suction pump.

Tube 5: Connected with tube containing 10 c c. of methyl red, as in tubes 1 and 3.

Tube 6: Connected with a 140 c c. flask in which was placed a tube containing 10 c c. of methyl red indicator, as in tubes 1 and 3.

Tubes 1 and 2 were connected with a water power suction pump and mercury gage. The tubulures were sealed when the gage indicated a vacuum of 2 mm. Tubes 3 and 4 were connected with the suction pump and sealed when the mercury registered 180 mm. Tubes 5 and 6 were closed at ordinary atmospheric pressure.

The changes in reaction observed in the indicator tubes, the appearance of the culture tubes, and the amount of oxygen present are recorded in table 2. The P_H determinations were made by comparing the indicator tubes with color standards prepared by Medalia's method.[6]

TABLE 2

RESULTS IN EXPER. 10

Tube	Culture Tubes						Indicator Tubes			Amount of Air
	24 Hours		48 Hours		10 Days		24 Hours, P_H	48 Hours, P_H	10 Days, P_H	
	Slant	Butt	Slant	Butt	Slant	Butt				
1	acid	acid	acid	acid	acid	acid	yellow	yellow	yellow	2 mm.
2	acid	acid	acid	acid	acid	acid	6.0	6.0	6.0	2 mm.
3	acid	acid	acid	acid	acid	acid	5.2	5.2	5.2	180 mm.
4	acid	acid	acid	acid	acid	acid	yellow	yellow	yellow	180 mm.
5	alk.	acid	alk.	⅕ alk. ⅘ acid	alk.	¼ alk. ¾ acid	5.0	5.0	5.0	33.5 c c.
6	alk.	acid	alk.	¼ alk. ¾ acid	alk.	alk.	5.4	5.2	5.0	155.0 c c.

In tube 1 insufficient acid was produced to give a red color with methyl red; in tube 4 the volatile acids produced carried the reaction beyond the acid end point of brom cresol purple.

EXPER. 11.—This experiment showed the influence of different quantities of dextrose, the oxygen content being approximately the same.

Typhoid double-sugar tubes containing different amounts of dextrose, from 0.00% to 0.5%, as indicated in table 3, were connected with tubes containing 10 c c. of sterile water and 0.8 c c. of 0.02% aqueous solution of methyl red. A Russell tube inoculated with Bact. alkaligenes and connected with a methyl red tube, was included in this series. The tubes were incubated for 3 days.

The results are recorded in table 3.

EXPER. 12.—Three Russell tubes were inoculated, respectively, with Bac. alkaligenes, Bac. typhosus, and Bac. paratyphosus B. Two sugar-free tubes were inoculated, respectively, with Bac. typhosus and Bac. paratyphosus B. An uninoculated control was included in the series. Each of the 6 tubes was connected with a tube containing 10 c c. of sterile water and 0.8 c c. of 0.02% aqueous solution of brom cresol purple. Tubes were then connected with suction pump as in exper. 9. In exper. 9 tubes 1 and 2 were sealed when the mercury gage registered 2 mm.; in this experiment suction was continued for

[6] Jour. Bacteriol., 1920, 5, p. 441.

20 minutes after the gage registered 2 mm. After 2 days' incubation the following reactions were observed in the indicator tubes.

1. Uninoculated control—P_H 6.6.
2. Alkaligenes, Russell—P_H 6.6.
3. Typhoid, Russell—P_H 6.2.
4. Typhoid, sugar-free—P_H 6.6.
5. Paratyphoid, Russell—P_H 5.2 or less (yellow).
6. Paratyphoid, sugar-free—P_H 6.6.

To demonstrate that the slight change in tube 3 and the absence of any changes in tubes 2, 4, and 6, were due solely to lack of oxygen, the tubulures were broken after the 48 hour readings were taken, air was allowed to enter the tubes, and the tubulures were again sealed. After 24 hours all indicator tubes, except the control, were yellow, which signifies a P_H of 5.2 or less.

The changes produced in the indicator tube by the paratyphoid Russell culture are not difficult to explain, since this organism is known to produce CO_2

TABLE 3

RESULTS IN EXPER. 11

Tube	Dextrose Content, %	Amount of Air, C c	Reaction-Indicator Tubes		
			24 Hours, P_H	48 Hours, J_H	72 Hours, P_H
1	0.00	33.5	5.0	4.8	4.8
2	0.02	34.0	5.0	4.8	4.8
3	0.04	32.0	5.2	5.2	5.2
4	0.06	31.5	5.4	5.2	5.2
5	0.08	32.0	5.2	5.2	5.2
6	0.1	33.5	5.2	5.0	5.0
7	0.2	32.0	5.0	5.0	5.0
8	0.3	32.5	5.2	5.0	5.0
9	0.4	30.0	5.6	5.4	5.4
10	0.5	33.5	5.0	5.0	4.8
Alkalig.	0.1	32 5	5.2	5.0	5.0

by the fermentation of dextrose. The sugar-free culture produced no change in the indicator tube. The production of a small amount of volatile acid by the typhoid Russell culture, with no acid formation by the sugar-free culture, may be explained in one of three ways: According to the opinion expressed by Nichols and Wood,[4] CO_2 is one of the end products of the fermentation of dextrose by the typhoid bacillus. Assuming this to be true, the slight acidity in tube 3 could thus be accounted for. Considering the slight acidity observed here and the marked acidity seen in the butt of a Russell tube, which they also attribute to CO_2, this explanation seems hardly plausible.

On the other hand, it is generally believed that in fermenting dextrose, especially under anaerobic conditions, the typhoid bacillus obtains oxygen from the dextrose molecule. If this be true, some of the oxygen thus obtained may be utilized by the organism in the same way that atmospheric oxygen is ordinarily utilized, CO_2 being an end product of this particular phase of metabolism. The process is quite distinct from the one attributed to gas producing organisms by virtue of which they ferment dextrose with the formation of H.COOH, then split this formic acid molecule into CO_2 and H_2.

The third possibility is that the slight acidity in the indicator tube is due, not to CO_2, but to other acids which, under the conditions existing within the tube, escape from the medium and produce the changes observed. This view

receives some support in the results reported by Harden.[7] He cultivated typhoid bacilli in 2.0% dextrose peptone water, in an atmosphere of nitrogen, and found the end products to consist of alcohol and lactic, acetic, succinic, and formic acids, with no CO_2.

Considering the small amount of acid produced by tube 3 and the findings already reported in regard to the effects of oxygen and of dextrose on volatile acid production, we are inclined to favor the second hypothesis.

The changes in the indicator tubes after atmospheric oxygen was permitted to enter show, again, the important part played by oxygen in volatile acid production.

Conclusions.—The formation of volatile acids in a typhoid Russell tube appears to be influenced to a much greater extent by the amount of oxygen present than by the dextrose content of the medium, so far as the effect of these acids on the indicators is concerned.

In Exper. 9 the oxygen supply was greatly restricted and no volatile acids were demonstrable, although the dextrose was completely fermented. In Exper. 10, the amount of dextrose being the same in all tubes, the degree of acidity indicated varied directly with the amount of oxygen present. In Exper. 11, the amount of oxygen being approximately the same in all tubes, the degree of acidity indicated varied but little, and these variations were not at all in accord with the variations in the amount of dextrose present. Bact. alkaligenes, which does not ferment either of the sugars in a Russell tube, produced quite as much volatile acid as did the typhoid bacillus. These findings indicate that when a fairly liberal supply of oxygen is provided, the amount of dextrose in the medium has practically no effect on volatile acid production. Exper. 12 furnishes additional evidence that volatile acids are produced in proportion to the amount of oxygen available for use by the organism.

The evidence thus far presented supports Russell's original statement that the oxygen supply is the factor which controls the appearance of a 24-hour typhoid Russell tube. In every instance in which the oxygen supply was adequate the tube showed an alkaline slant and an acid butt. In every instance in which the oxygen supply was greatly restricted, slant and butt were acid.

It still remains to be demonstrated which of the metabolic proccesses taking place within the typhoid Russell tube is responsible for the characteristic changes in this medium. It must be one, evidently, that is dependent on the oxygen supply. Russell's idea that it is fermentation of dextrose in the butt and absence of fermentation in the slant is not

[7] Jour. Chem. Soc., 1901, 79, p. 610.

tenable, since it has been shown that this process takes place more rapidly on the slant than in the butt and is independent of the oxygen supply.

There are, however, three other factors concerned in the mechanism of the typhoid Russell tube, all of which are dependent on the oxygen supply for their consummation. These are, volatile acid production, alkali production, and the rate and luxuriance of growth of the organism. The three are mutually interactive to a considerable degree and are manifestations of nitrogenous metabolism.

The changes in a Russell tube can hardly be attributed to volatile acids since the very conditions that favor maximum acidity in the tube are those under which volatile acid production is at its minimum. When the organisms are grown anaerobically the slant is acid regardless of whether the small quantities of volatile acids that may be formed under these conditions remain in the tube (Exper. 3 and 4) or escape (Exper. 5 and 6). On the other hand, the retention of volatile acids within the tube does not prevent the slant and the upper half of the butt from becoming alkaline, provided free oxygen is available (Exper. 8). Also, the behavior of a slant in which the acidity is due to volatile acids is quite different from that of a slant rendered acid by the products of dextrose fermentation (Exper. 7).

In the experiments recorded it will be noted that when oxygen was withheld the slants became acid, when oxygen was plentiful the slants were alkaline. It has already been shown that fermentation of dextrose is independent of the oxygen supply. It seems fair to assume, therefore, that approximately the same amount of acid was formed from the 0.1% of dextrose in all of the tubes, whether aerobic or anaerobic. The anaerobic tubes remained acid because an insufficient amount of alkali was formed to neutralize the acidity. The aerobic tubes showed an alkaline slant because conditions were favorable for alkali production, and the amount produced was sufficient to neutralize the acid completely. The characteristic changes in a 24-hour typhoid Russell tube are due to the fact that the typhoid bacillus, provided with an adequate amount of oxygen, can produce enough alkali within 18 to 21 hours to neutralize all of the acid formed in the slant by the fermentation of 0.1% of dextrose. It cannot produce enough alkali to neutralize all of the acid formed in the butt because the amount of oxygen in the butt is inadequate.

The rate and luxuriance of growth of the organism are closely allied with alkali production. In all tubes in which the oxygen supply is

greatly reduced, growth on the slant is hardly visible. In tubes with plenty of oxygen, growth is luxuriant and there is little difference in this respect between a Russell tube and a sugar-free tube when both are inoculated with old stock cultures; the Russell slant is, itself, sugar-free after 15 hours. The initial growth of the organism is, no doubt, stimulated by the small amount of dextrose present, but at the end of 24 hours conditions in the two tubes are nearly equal.

The remaining experiments have to do with the changes that take place in a Russell tube after 24 hours. The slant remains alkaline; the butt gradually becomes alkaline from above downward and is completely alkaline within 5 to 8 days. Nichols and Wood [4] attribute this to a diffusion downward of alkaline substances produced on the slant. In our opinion it is a continuation of the process already observed on the slant. The organism utilizes the oxygen as fast as it penetrates into the butt and produces alkali. Within from 5 to 8 days a sufficient amount of oxygen enters the butt to enable the organism to produce enough alkali for the complete neutralization of the acid.

E. Experiments were performed to determine whether or not alkaline substances produced in the slant of a typhoid Russell tube diffuse downward into the butt.

Exper. 13.—Russell and sugar-free slants, both indicators, were prepared, placed in the incubator in inverted position over night, then right side up for 6 hours. The medium then presented a dry slant surface and had no water of condensation. In one set of tubes the upper four fifths of the slant surface was inoculated with a loopful of typhoid bacilli from a 24-hour slant agar culture. These slants showed the characteristic alkalinity, but there was no change in reaction in the butts of the tubes during the 10 days they were kept under observation. The tubes in the duplicate set were inoculated, slant and butt, and showed the usual changes, becoming completely alkaline on the 7th day.

Conclusion.—Alkaline substances produced in the slant of a typhoid Russell tube do not diffuse downward into the butt in quantities sufficient to cause a color change in the indicator.

F. Experiments were performed to determine the influence of the oxygen supply on the changes observed in the butt of a typhoid Russell tube.

Exper. 14.—Four sets of 4 tubes each, Russell and sugar-free medium, Andrade and phenol red indicators, were inoculated, slant and butt. The tubes of one set were connected with alkaline pyrogallol tubes as soon as inoculated. The tubes of the other 3 sets were connected with alkaline pyrogallol after incubation of 7 hours, 18 hours, and 42 hours, respectively.

At the end of 24 and 48 hours the tubes that were connected immediately showed marked acidity in slant and butt in both Russell tubes, no change in

sugar-free Andrade tube, neutral slant with slight acidity in the butt of the sugar-free phenol red tube. The tubes that were connected after 7 hours showed, at the end of 24 and 48 hours, the same changes that were observed in the first set of tubes, with the exception of the sugar-free phenol red tube which showed a moderately alkaline slant.

After the 48-hour observations were made, these two sets of tubes were disconnected from the alkaline pyrogallol tubes. Twenty-four hours later they presented the appearance of normal typhoid Russell tubes; 10 days later all tubes in these two sets were completely alkaline.

The Russell tubes that were connected with alkaline pyrogallol after they had incubated 18 hours and 42 hours showed alkaline slants and acid butts at the time the connection was made. Since there was no dextrose remaining in the slants (exper. 1), the alkaline reaction of the slants persisted. Alkali production continued for a time in the butts of the tubes, so that the final results in the Andrade tubes were: (a) in the 18-hour tube, butt ⅓ alkaline, ⅔ acid; (b) in the 42-hour tube, butt ½ alkaline, ½ acid. No further change occurred during ten days. In the phenol red tubes the butts remained acid for 10 days. The 18-hour and 42-hour sugar-free Andrade tubes showed no changes in reaction. The cessation of alkali production in the sugar-free phenol red tubes corresponded with that in the Russell phenol red tubes.

These results illustrate the effects of shutting off the supply of oxygen at different time periods. Soon after this is done, alkali production in the butt ceases.

EXPER. 15.—Loeffler's alkaline methylene blue, 0.03 c c., was added to Russell and sugar-free Andrade tubes; they were then placed in the boiling water bath until the methylene blue was decolorized, then slanted in the icebox. When solid, the slants were green, the butts were colorless. Russell and sugar-free tubes were inoculated, slant and butt; duplicate tubes were left uninoculated.

Slants of all tubes remained green throughout the experiment. The butt of the uninoculated Russell tube regained its green color, from above downward, after 6 days; in the uninoculated sugar-free tube the color returned after 4 days. The butt of the inoculated Russell tube showed the characteristic changes: strongly acid at first, gradually becoming alkaline from above downward, with complete alkalinity on the 8th day. The methylene blue remained almost entirely decolorized; on the 8th day the green color had descended only 8 mm. into the butt. This was interpreted as indicating that the organisms were still living and were capable of utilizing all of the oxygen that entered the butt. After the tenth day the medium had dried out to such an extent that it began to split and to shrink away from the walls of the tube. The green color then returned rapidly. The methylene blue in the butt of the inoculated sugar-free tube showed practically the same changes as in the Russell tube.

Conclusions.—Alkali production in the butt of the tube is dependent on the oxygen supply. It goes on to completion when oxygen is provided; it stops when oxygen is withheld. The results with methylene blue indicate that the oxygen is actually utilized by the organisms, since the decolorized dye regains its color in from 4 to 6 days when no organisms are present.

SUMMARY

The appearance of a 24-hour typhoid Russell tube is dependent on the amount of oxygen available for use by the organisms. This is in accord with one part of Russell's original explanation.

The typhoid bacillus ferments all of the dextrose in a Russell tube within 21 hours, regardless of the oxygen supply. This is contrary to Russell's conception of the way in which oxygen produces its effects.

Volatile acid production, although dependent on the oxygen supply, has no direct bearing on the appearance of a typhoid Russell tube. These acids are respiratory, not fermentative, in origin. This is not in accord with the views expressed by Nichols and Wood.

The characteristic changes in a 24-hour typhoid Russell tube are due to the influence of oxygen on alkali production. In the slant, where oxygen is plentiful, sufficient alkali is produced within from 18 to 24 hours to neutralize all of the acid formed by the fermentation of 0.1% of dextrose, and the slant is alkaline. In the butt, where oxygen is scanty, the amount of alkali produced in 24 hours is not sufficient to neutralize all of the acid formed by the fermentation of 0.1% of dextrose, and the butt is acid.

The alkaline reaction seen in the butt after from 5 to 8 days is due to slow, continued, alkali production within the butt, not to a diffusion downward of alkaline substances produced in the slant.

STREPTOCOCCI OF SOURING MILK WITH SPECIAL REFERENCE TO STREPTOCOCCUS LACTIS *

STUDIES OF THE STREPTOCOCCI. IX

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An attempt has been made in some of the preceding papers of this series to show the characteristic species of streptococci which are found in the udder, mouth, and feces of cows and to name the principal species. The sources of the streptococci were such that we might expect the majority of this type of organism introduced into milk to come from them.

Much confusion exists as to the species of streptococci found in milk at different stages in its history, and there is doubt as to whether the identity of the typical streptococcus of sour milk is generally appreciated.

The medical bacteriologists usually think of streptococci in milk as indicating a suppuration in the udder or as evidence of some definite contamination, while on the other hand, the dairy bacteriologists usually think of the harmless lactic acid organism. These two points of view are often displayed in attempts to interpret the significance of streptococci in milk.

No claim is made that the results presented in this paper will clear up all the confusion which now exists, but we hope that they will contribute to a better understanding of this interesting subject.

OUTLINE OF THE INVESTIGATION

The milk examined represented an average grade of market milk. It was not produced on special farms or by special methods. Streptococci were isolated by Paul W. Clemmer, formerly of these Labora-

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* It is believed that Streptococcus lactis is the name which should be applied to the typical Streptococcus of sour milk instead of Streptococcus lacticus (Kruse). Although Kruse was the first to give this name, Lister really discovered the organism which he termed Bacterium lactis. This organism he described as oval shaped and not so long as bacteria in general. On grounds of priority it is believed the organism should therefore be called Streptococcus lactis.

tories, by plating on dextrose extract agar plates, then examining microscopically, and finally purifying by replating.

The first set of 101 cultures was obtained from milk with an acidity of from 0.18 to 0.25% calculated as lactic acid, and represented 40 samples from 40 farms. These were composite samples each made up from several cans from each farm. In all cases, the samples were held a few hours at room temperature to allow a slight development of acidity.

The second set of 22 cultures was isolated from 8 composite samples from 8 farms, which were allowed to stand at room temperature until they had reached an acidity of from 0.30 to 0.49%.

The third set of 89 cultures was isolated from 29 composite samples of milk which had been held at room temperature until an acidity of 0.6% or more of acid had developed. The composite samples of milk were in this case obtained by taking a few c.c. of milk from the cans of many different farmers as they brought their milk to the receiving station.

It will be seen that the streptococci were isolated from milk having three distinct ranges in acidity, and they, of course, represented the majority of the streptococci present in the milk at the time of the examination. Since it was found that the streptococci of the udder, mouth and feces of the cow would grow readily on the dextrose agar, they would have been isolated by the method used if present in sufficient numbers to represent the majority flora.

The streptococci of milk with acidities from 0.18 to 0.25%

Since streptococci are usually present in the udders of even apparently normal cows, one might expect to find them growing in milk together with those from cow feces and other material from the body of the cow which contaminates milk to a greater or less degree. With these streptococci, usually if not always introduced, there are those coming from unsterilized utensils and other minor sources of contamination.

In earlier papers of this series we have described the characteristic streptococci of the udder, feces and mouth of the cow and have shown that their characteristics are sufficiently definite to permit their recognition. It should be, therefore, a relatively easy matter to trace their developments in milk, provided they are present in sufficient number to play an important part in its history.

As stated in the outline of this investigation, the first set of 101 streptococci were isolated from 40 samples of market milk having acidities ranging from 0.18 to 0.25%.

The cultural characteristics were determined by the methods described in an earlier paper by Ayers and Mudge,[1] except that the following medium was used for fermentation tests in place of yeast broth: 15 gm. peptone (Bacto), 3 gm. meat extract, 5 gm. test substance, 1,000 c c. distilled water. Reaction adjusted to P_H 7.5.

We are indebted to Dr. Rupp of these laboratories for making the tests for ammonia and the hydrolysis of sodium hippurate.

Instead of recording the cultural characteristics of every culture, similar cultures have been grouped together as shown in table 1. It must be realized that not every culture gave the exact P_H values here given in the fermentation test. There was a slight variation, but the figures represent the general limits reached. For convenience in discussion of the results some of the groups of cultures have been given a number.

A study of the results in table 1 shows some interesting facts. Instead of the streptococci of the udder, feces or mouth of the cow being the predominating type, it is seen that Streptococcus kefir holds that position. Of the 101 streptococci isolated, 68 were found to be varieties of the streptococcus mentioned.

Streptococcus kefir.—This streptococcus, so named by Migula,[2] was first isolated by Freudenrich[3] from kefir, and has been shown by Evans[4] to be common in cheese. It is characterized by its ability to form gas from sugar, as has been shown by Sherman.[5] This streptococcus produces as much gas as the colon bacillus when measured in Eldredge tubes but, as this work of Ayers, Rupp and Mudge[6] has shown, this gas formation is not demonstrated in Smith tubes. Streptococcus kefir, as was observed by Evans[4] usually ferments raffinose, and this characteristic was common to 64 of 68 cultures in our collection. Four of the cultures did not ferment it.

It will be of interest to note at this point that in our studies of streptococci from the udder, feces and mouths of cows we have never encountered a gas-forming streptococcus. If present in such places, they were there in such small numbers as to escape detection.

[1] Jour. Infect. Dis., 1922, 31, p. 40.
[2] System der Bacterien, 1900, 2.
[3] Centralbl. f. Bakteriol., 1897, 3, p. 47.
[4] Jour. Agric. Res., 1918, 13, p. 235.
[5] Jour. Bacteriol., 1921, 6, p. 127.
[6] Jour. Infect. Dis., 1921, 29, p. 235.

TABLE 1

CULTURAL CHARACTRISTICS OF STREPTOCOCCI HAVING AN ACIDITY FROM 0.18 TO 0.25%

Group	Number of Cultures	Morphology	Hemolysis	Methylene Blue Reduced	Litmus Milk Reaction		Janus Green Reduced	P_H in Medium with Surface Tension of 42.8 Dynes	Final P_H in Fermentations							C c. of CO_2 from		NH_3—N from Peptone. Mg per 100 C c. Excess over Control	Sodium Hippurate Hydrolyzed		
					30 C.	10 C.			Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin	Peptone	Dextrose				
	64	Chains of 2-6 sometimes more	Gamma some green sometimes weak alpha	Most always negative	Acid not coagulated	Slightly acid	..	Range 5.2-5.5 Av. 5.45	4.6 +	4.6 +	4.7 +	7.5 —	7.5 —	4.8 +	7.5 —	Range 0.0-0.97 —	Range 3.1-5.9 +	Range 0.0-0.28 —	—	Var. 1	Streptococcus kefir
	4	Chains of 2-6 sometimes more	Gamma some green	—	Acid not coagulated	Slightly acid	..	5.3-5.4 Av. 5.37	4.6 +	4.6 +	4.7 +	7.5 —	7.5 —	7.5 —	7.5 —	0.60-0.69 —	5.1-5.8 +	0.0-0.14 —	—	Var. ?	
1	7	Chains of 2-6 usually	Gamma green sometimes weak alpha	Typical	Typical	Typical	+	4.6-5.3 Av. 4.73	4.3 +	4.3 +	7.6 —	4.8 +	4.8 +	7.6 —	7.6 —	2.8-3.6 +	..... —	18.06-22.12 +	+	Var. A	Streptococcus lactis
2	2	Chains of 2-6 usually	Gamma green sometimes weak alpha	Typical	Typical	Typical	+	4.6	4.3 +	4.3 +	7.6 —	4.8 +	7.6 —	7.6 —	7.6 —	3.9-4.1 +	..... —	21.30-22.26 +	+		
3	6	Chains of 2-6 usually	Alpha weak	Typical	Typical	Typical	+	4.5-4.6 Av. 4.55	4.3 +	4.3 +	4.3 +	4.6 +	4.5 +	7.6 —	7.6 —	4.4-4.8 +	..... —	19.34-23.12 +	+		
4	1	Chains of 2-6 usually	Alpha weak	Typical	Typical	Typical	+	4.6	4.4 +	4.5 +	7.6 —	7.5 —	7.4 —	7.5 —	7.4 —	4.9 +	..... —	20.32 +	+		
5	3	Chains of 2-6 usually	Gamma green	Typical	Typical	Typical	+	5.0-5.6 Av. 5.26	4.3 +	4.5 +	7.4 —	7.5 —	7.5 —	7.4 —	7.4 —	0.0-1.20 —	0.0-0.80 —	0.0-0.14 —	1— 2+	Var. B	Streptococcus lactis
6	5	Chains of 2-6 usually	Gamma some green	—	Typical	Typical	+	5.2-5.8 Av. 5.35	4.4 +	4.5 +	4.4 +	4.5 +	7.4 —	7.4 —	7.4 —	0.05-0.40 —	0.0-0.10 —	0.0-0.70 —	1+ 4—		
7	3	Chains of 2-6 usually	Gamma	—	Typical	Typical	+	5.3-5.4 Av. 5.36	4.4 +	4.6 +	4.6 +	4.7 +	4.5 +	5.1 +	7.4 —	0.45-1.04 —	0.0-0.10 —	0.0-0.42 —	1+ 2—		
8	1	Chains of 2-6	Gamma green	—	Typical	Typical	+	5.4	4.4 +	4.4 +	4.4 +	4.5 +	4.5 +	7.4 —	7.1 —	0.30 —	0.0 —	0.0 —	—		
10	3	Numerous chains of 6–12 sometimes longer	Beta weak	—	Acid coagulated ⅓ decolorized	Very slight acid	+	5.6-5.8 Av. 5.66	4.5 +	4.8 +	4.6 +	5.5 +	7.4 —	7.4 —	7.6 —	4.2-4.6 +	..... —	22.44-22.98 +	+		
11	2	Chains of 2-6 usually	Gamma	—	Acid	Slight acid	..	5.4-5.5 Av. 5.45	4.5 +	4.6 +	4.6 +	4.8 +	4.8 +	7.5 —	7.5 —	4.4-4.5 +	..... —	26.48-27.74 +	+		

It should be remembered that the milk from which these streptococci were isolated was not absolutely fresh, for it had been held a few hours to allow a slight acidity to develop, but not long enough to be sour to the taste. Evidently Streptococcus kefir is introduced into milk in small numbers but grows with sufficient rapidity to form the majority streptococcus flora during the early period of bacterial growth.

At this point we must call attention to the rather striking resemblance between the predominating variety of Streptococcus kefir and Streptococcus bovis, the characteristic streptococcus of cow feces as described by Ayers and Mudge [7] in a previous paper of this series. From the reaction of these organisms as shown in table 2, it seems evident that Streptococcus kefir might easily be mistaken for Streptococcus bovis unless the gas test is applied. Such a mistake would be misleading to anyone considering the raffinose fermenting streptococci as cow fecal types and interpreting their presence as evidence of fecal contamination. Although more or less contamination by cow feces must occur under the usual conditions of milk production, a glance at table 1 reveals the fact that Streptococcus bovis was not present among the "majority streptococci." This is not so strange because this species, so far as our experience goes, does not grow rapidly in milk, and it is therefore probably rapidly overgrown.

Referring again to table 1, it will be noted that Streptococcus mastiditis was not found among the predominating streptococci. Since this organism is the most common streptococcus of the cow's udder and has been found in a large percentage of the animals examined by Ayers and Mudge [1] and others, they are undoubtedly present in all milk. Three cultures, group 10, bore the closest resemblance to the udder streptococcus but differed in as far as they grew at 10 C. and produced volatile acids in milk. Streptococcus mastiditis does not grow at 10 C. and produces only a small amount of volatile acid when grown in skimmed milk.

Jones,[8] in studying the source and significance of streptococci in market milk, found that most of the organisms isolated belonged to the group of udder streptococci. This is not necessarily a disagreement with our results, because the market milk examined by Jones was distinctly different from that we have studied. He isolated his cultures from a low count certified milk purchased at random. This was really a special milk produced under special conditions where contamination

[7] Ibid., 1923, 33, p. 155.
[8] Jour. Exper. Med., 1920, 31, p. 347.

appreciable numbers. Doubtless in the future, when streptococci as a whole group are better understood, the organism will be found to be more commonly distributed.

The papers of many investigators, and often textbooks, carry statements to the effect that the lactic-acid streptococcus is identical or at least closely allied to the streptococci from pathologic sources, feces, skin and mouth of the cow, and that they can be readily isolated from some of these sources. Doubtless a clearer conception of the cultural characteristics of the lactic streptococci will cause such general statements to be modified. The object in bringing up this discussion at this time is to free the mind of the reader from what appear to be misleading facts which, in the light of recent investigations, are perhaps not entirely correct.

In 1884, Heuppe [12] isolated an organism which he thought caused the souring of milk and called it B. acidi lactici. Later, Günther and Theirfelder [13] (1894-1895) found an organism in sour milk which was different from the one described by Heuppe; this was B. lactici acidi (Leischmann).

Kruse,[14] in 1893, believed that B. acidi lactici (Heuppe) could be included in a group with B. aerogenes, and this subject was finally cleared up by Heinemann [15] in 1906. Kruse gave the name Streptococcus lacticus to the typical sour milk organism which he believed to be a streptococcus and not a short rod, and so B. lactici acid became known to many as Streptococcus lacticus. It will be remembered that Lister was the first to isolate the lactic ferment from milk, and he called it Bacterium lactis. He said the organism was oval shaped and not so rod shaped as bacteria in general. Because of his work, Lohnis [16] believes that on the grounds of priority the typical lactic-acid organism of milk should be called Streptococcus lactis. This is the name used by Jensen [17] in his classification, and we believe it to be the correct term.

No attempt has been made to review all the work which has led to a large number of descriptions of species of lactic bacteria, the most common of which are shown in the following list: Bacterium lactis (Lister), B. lactici acidi, B. lactis acidi (Leischmann), Sphaerococcus

[12] Mitt. K. Gsndhtsamt., 1884, 2, p. 367.
[13] Hyg. Rundschau, 1894, 4, p. 1105; Arch. f. Hyg., 1895, 25, p. 164.
[14] Centralbl. f. Bakteriol., I. O., 1903, 34, p. 737.
[15] Jour. Infect. Dis., 1906, 3, p. 173.
[16] Centralbl. f. Bakteriol., 1909, 22, p. 553.
[17] Mem. Acad. roy. d. Sc. et d. Lettres de Danemark, Section d. Sc., 1919, 5, No. 2.

acidi lactic (Markmann), Bacterium Guentheri (Lehmann & Neumann), Streptococcus Guntheri (Lehmann & Neumann), Streptococcus lacticus (Kruse) and Streptococcus lactis (Lohnis).

It is now generally conceded that the typical lactic organism of sour milk is a streptococcus, usually called Streptococcus lacticus, but occasionally some of the other synonyms are used by those who believe it to be a short rod. Although there is quite a general agreement that the organism is an oval streptococcus, usually in pairs or short chains, its cultural characteristics are not sharply defined.

In studying species of bacteria, collections should be obtained from their natural habitat, and for this reason the cultural characteristics of streptococci isolated from sour milk which represent the majority flora might properly be considered the true lactic acid streptococcus. Sherman and Albus [18] have studied a collection of cultures isolated, according to this plan, and they showed plainly the value of certain tests, and at the same time outlined the characteristics of Streptococcus lacticus. Their work has done much toward clearing up the confusion which exists as to the identity of the typical streptococcus of sour milk. They pointed out, among other things, that the typical lactic type grows at 10 C., reduces methylene blue, does not ferment saccharose and produces a quick reduction of litmus when in sterile milk. Esten,[19] in 1909, pointed out that one of the characteristics of the lactic acid organism is its ability to reduce litmus milk, and that the first reaction is that of decolorization. Hastings [20] also noted this fact, and Sherman and Albus [18] have shown that this property, particularly that of reduction before visible acid change, is a valuable one for diagnostic purposes, especially when correlated with other characteristics. At this point it is necessary to explain the Janus green reduction test which has been applied in our studies.

Janus Green Reduction Test.—The reduction of Janus green has been found to be valuable in the classification of streptococci. Although space does not permit a detailed description of the experiments carried on to determine the best methods for making the reduction test, a description of the test as actually performed is necessary.

The color change from blue green to red when Janus green, diethylsafranineazodimethylaniline, is reduced is probably due to the breaking up of the color molecule into safranine and dimethylaniline. The loss

[18] Jour. Bacteriol., 1918, 3, p. 153.
[19] Conn. Agri. Exper. Station Bull. 59.
[20] Marshal, C. E.: Microbiology, 1911, p. 308.

of the red color is probably due to further reduction of safranine to the colorless leukobase which is easily reoxidized by air.

Experiments indicate that many and perhaps all streptococci can reduce Janus green under suitable conditions. They probably all have reducing power, which however varies in degree. The test has been arranged so that reduction was obtained with certain species of streptococci and not with others, and for successful results the test must be used as described.

To make the test, cultures are grown in the following medium:

Infusion broth	
Peptone (Parke-Davis)	1.0%
Dextrose	0.5%
Reaction adjusted, before sterilization, to P_H 7.5	

To each culture, containing about 8 c c. of medium, after incubation for from 18 to 24 hours, 0.1 c c. of sterile 0.5% solution of Janus green is added. Janus green cannot be sterilized in culture medium by heat without undergoing reduction, but it can be sterilized by heat in aqueous solution.

Reduction usually starts within 30 minutes, and the reaction is usually shown within 3 hours if the streptococci have the ability to reduce the dye under the conditions of the test, while examination after 3 hours usually shows whether or not reduction will occur; holding for longer periods may show a greater degree of reduction. The first color change is to purple then to red, and if the reduction is sufficiently strong the medium is decolorized.

It is best to select the optimum growing temperature of the organism to be tested for the incubation temperature. Cultures of Streptococci lactis were grown at 30 C., and bovis, mastiditis and pyogenes at 37 C.

The test must be made in the medium described and the cultures be from 18 to 24 hours old. Change in the medium influences the results and may result in negative tests when they should be positive. The age of the culture at which the test is made is important, as it may be positive after 20 hours and weak or negative in 48 hours.

The reduction of Janus green seems to be closely connected with the vital activity of the bacteria cells and is influenced by many things which disturb this activity. While many of these have been studied, such as the reduction of surface tension and influence of heat, the data give no clear conception of the mechanism of the process. Much more work needs to be done on the Janus green test.

Although the reduction of Janus green is subject to the influence of various conditions, the test when properly applied under controlled conditions seems to give more constant results than the reduction of methylene blue.

The value of the Janus green reduction test as a means of separating streptococci is shown in table 3. The test has been applied to different groups of streptococci in our collection, each of which consists of quite a large number of cultures. It will be noted that Streptococcus lactis incubated at 30 C. reduces Janus green, while Streptococcus bovis, mastiditis, and pyogenes do not when incubated at 37 C. Streptococcus kefir may reduce slightly under some conditions and can be readily differentiated by the reduction of CO_2 from dextrose.

TABLE 3

REDUCTION OF JANUS GREEN BY DIFFERENT STREPTOCOCCI

Name	Group No.	Number Cultures	Action on Janus Green after 3 Hours			
			Red. Nearly or Partly Decolorized	Red	Blue Green and Red	Blue Green No Change
Streptococcus lactis Var. A	1	59	53	2	4	0
	2	21	16	3	2	0
	3	13	10	0	3	0
	4	1	1	0	0	0
	9	7	5	1	1	0
Streptococcus lactis Var. B	5	3	3	0	0	0
	6	6	0	0	6	0
	7	3	0	0	3	0
	8	1	0	0	1	0
Streptococcus bovis	—	68	0	0	0	68
Streptococcus mastiditis	—	48	0	0	0	48*
Streptococcus pyogenes	—	32	0	0	0	32†

* Three cultures showed trace of red in sediment.
† One culture showed trace of red in sediment.

Reduction of Ammonium Molybdate.—The reduction of molybdate in a yellow culture medium, to a deep blue color, gives another possible test which may be useful in studying streptococci. We have not worked the test out completely because of the closing of this series of studies of the streptococci at this time, but it is felt that the test, although incomplete, will be of interest.

The medium used is the same as the one for the reduction of Janus green, and the incubation period is 24 hours at 30 C. In each tube of 8 c c. culture medium 2 c c. of a 5% solution of sterile ammonium molybdate is added. The reduction is shown in from 1 to 6 hours by

the appearance of a deep blue color, due probably to the formation of the lower oxides of molybdenum. As a rule, Streptococcus lactis shows the deep blue color in the lower half of the tube in about 3 hours. Streptococcus bovis, mastiditis and pyogenes are negative, or show a faint blue.

It is also possible to add the sterile molybdate solution before incubation, and in such cases a smaller amount of ammonium molybdate gives better results. The reduction of the molybdate is another complicated test which requires much more study. Levine and Jahr found that Streptococcus pyogenes showed no growth or reduction with molybdate, but from our experiments it appears that reduction can be obtained by all streptococci under suitable conditions. It does appear, however, that the test can be arranged to show differences in the degree of reduction, and for this reason it will probably be found to be valuable.

Besides this reduction, growth in mediums with lowered surface tension has been found to be useful.

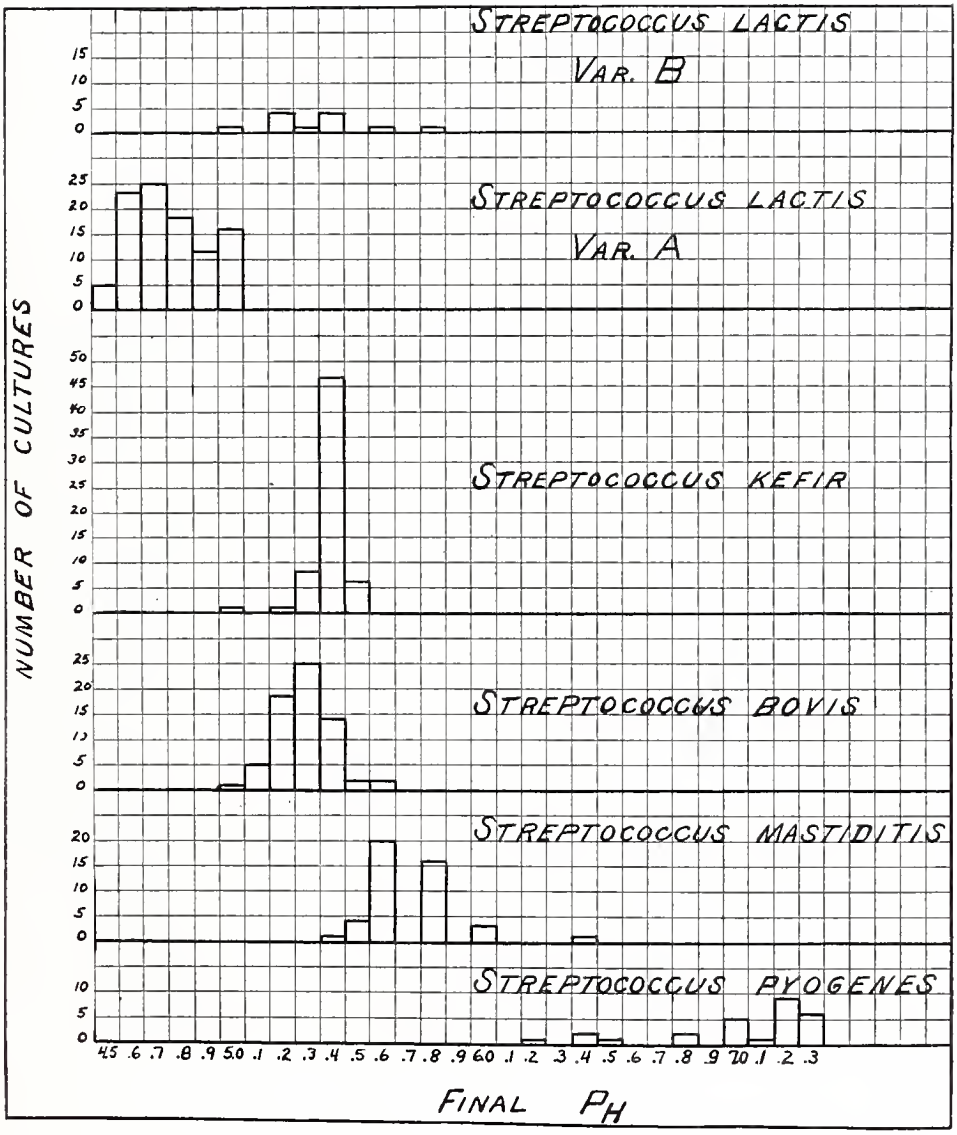
Growth at Lowered Surface Tension.—In some previous studies from these laboratories by Ayers, Rupp and Johnson the possibilities of using the influence of surface tension depressants as a means of classifying streptococci has been clearly indicated. As growth at lowered surface tension has been made use of in studying the streptococci of souring milk, it is desirable to present further information on this subject.

The test as recommended by Ayers, Rupp and Johnson was to measure the final P_H after 7 days' incubation at 30 C. in a medium consisting of infusion broth, Parke-Davis peptone 1%, dextrose 0.5% and reaction adjusted, before sterilization, to P_H 7.5 and the surface tension lowered to about 42.8 dynes with sodium glycocholate.

Growth in this medium as measured by the final P_H has been determined with a large number of cultures of different groups of streptococci, and the results have been plotted in fig. 1 so as to show the frequency with which cultures reach the same final P_H values.

It is clear from the results that the lactic acid streptococci show a much greater ability to grow at a surface tension of 42.8 dynes than the pathogenic pyogenes type. The other groups are not so widely separated on the basis of their final P_H in the surface tension medium, but Streptococcus mastiditis seemed to reach a final P_H between Streptococcus pyogenes and the other groups.

Although each culture in their respective groups did not reach exactly the same final P_H they did fall within a relatively close range. Perhaps more accurate P_H measurements would bring them closer together.



Final PH in dextrose broth with surface tension of 42.8 dynes.

From what we have seen of this test, we believe it indicates fundamental differences among streptococci and that it is useful as a means of studying them.

Culture Characteristics of Streptococcus Lactis.—It is perhaps unfortunate that the plan for the presentation of our results takes up the lactic streptococci of milk of low acidity before those representing the majority flora of sour milk. In our grouping, however, we have considered the cultures as a whole. It will be shown that the strain of the particular variety of Streptococcus lactis predominating in milk of low acidity holds a similar position in milk with an acidity of 0.6% or over.

From table 1 it will be seen that we have studied many cultural characteristics, among the most important of which are the typical reaction in litmus milk, growth at 10 C., reduction of Janus green, limiting P_H in medium with lowered surface tension, and positive or negative tests for CO_2 and ammonia from peptone. A typical reaction in litmus milk consists of the following:

Quick reduction of the litmus usually followed by rapid acid formation which results in a solid curd which is white except for a red ring about one-quarter inch deep at the top. This reaction occurs most always within 24 hours when cultures are incubated at 30 C. Many investigators are inclined to believe that rapid acid formation is one of the characteristics of Streptococcus lactis. We do not, however, believe that the rate of acid formation is particularly important, for it is often slowed down when pure cultures are kept in culture mediums. The exclusion of streptococci from the lactic group because of slow acid formation will doubtless exclude many which should be included. The typical reaction, when correlated with other things, is, we believe, characteristic of the lactic streptococci. This correlation has not been seen with any cultures from the udder, mouth or feces of cows. Esten [19] observed this typical reduction among streptococci from the mouth of cows, as has Rogers and Dahlberg.[21] Perhaps these cultures were true lactic acid streptococci.

It appears to us that the reduction of Janus green, as in the test previously described, is a little more definite than the reduction of litmus, because many species of streptococci reduce litmus slowly although they do not reduce Janus green as the test is arranged. However, neither the quick reduction of litmus nor Janus green is by itself of value for diagnostic purposes. These tests must be correlated with other reactions. For example, litmus and Janus green are quickly reduced by many of the streptococci of the human mouth, but these organisms differ so in other reactions that they can be readily separated from the lactic group.

[21] Jour. Agric. Res., 1914, 1, p. 491.

Correlated with powerful reduction and growth at 10 C. is the ability of the lactic streptococcus to reach a low final P_H in a medium, the surface tension of which has been reduced to 42.8 dynes with sodium glycocholate. This difference in final P_H between the lactic streptococci and other groups in our collection has been shown in fig. 1. It is the positive correlation between these three characteristics which appears to separate Streptococcus lactis from other groups of streptococci.

Going back to table 1 again, it may be seen that 28 of the streptococci of the 101 isolated from milk having acidities from 0.18 to 0.25% were varieties of Streptococcus lactis. The blood plate reaction usually showed the gamma type, generally with green coloration. These reactions were not particularly sharp, and in some cases weak alpha reactions were noted. No beta (hemolytic) reactions were observed among any of the cultures of Streptococcus lactis in our collection. There appear to us to be two varieties, based on the production of CO_2 and NH_3 from peptone, with several strains of each variety. As has been shown by Ayers, Rupp and Mudge,[6] the production of CO_2 and NH_3 from peptone appears to be an important characteristic of certain streptococci and is particularly valuable when correlated with other reactions. It is of further interest to observe that Streptococcus lactis var. A in the lowered surface tension medium reached a final P_H of about 4.7, while var. B reached a lower hydrogen-ion concentration of about P_H 5.3.

As will be shown later, group 1, which consisted of 7 cultures, appears, so far as our results are concerned, to be the common strain of the predominant Streptococcus lactis type of sour milk.

Sherman and Albus[18] found that the great majority of their cultures from sour milk did not ferment saccharose, and in this respect their results agree with ours. Most of their cultures did not ferment mannite or salicin. The majority of their cultures would agree therefore with group 4 or 5 of our collection. It is impossible to say which one, because they did not measure CO_2 or NH_3 production by the streptococci.

Jones,[22] in a study of the streptococci occurring in sour milk, found that the lactic group consisted of at least three species: one did not ferment saccharose but fermented mannite; another fermented saccharose and mannite; while a third fermented saccharose but not mannite.

[22] Jour. Exper. Med., 1921, 33, p. 13.

TABLE 4

CULTURAL CHARACTERISTICS OF STREPTOCOCCI FROM MILK WITH AN ACIDITY FROM 0.30 TO 0.49%

Group	Number of Cultures	Morphology	Hemolysis	Methylene Blue Reduced	Litmus Milk Reaction		Janus Green Reduced	P_H in Medium with Surface Tension of 42.8 Dynes	Final P_H in Fermentations							C c. of CO_2 from		NH_3—N from Peptone Mg. per 100 C c. Excess over Control	Sodium Hippurate Hydrolyzed	
					30 C.	10 C.			Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin	Peptone	Dextrose			
1	9	Chains of 2-6 usually	Gamma green sometimes weak alpha	+	Typical	Typical	+	Range 4.5-4.7 Av. 4.62	4.3 +	4.3 +	7.6 —	4.8 +	4.8 +	7.6 —	7.6 —	Range 3.0-4.4 +	Range . . . . —	Range 20.26-21.44 +	+	Streptococcus lactis Var. A
9	3	Chains of 2-6 usually	Gamma green	+	Typical	Typical	+	4.5-5.4 Av. 4.9	4.3 +	4.3 +	4.3 +	4.6 +	7.6 —	7.6 —	7.6 —	2.3-4.7 +	. . . . . —	20 46-22.44 +	+	
6	1	Chains of 2-6 usually	Gamma green	—	Typical	Typical	+	5.4	4.4 +	4.5 +	4.4 +	4.5 +	7.4 —	7.4 —	7 4 —	0.45 —	6 0 —	0.0 —	—	Streptococcus lactis Var. B
	8	Chains of 2-6 usually	Gamma some green	—	Acid not coagulated	Slight acid	. .	5.3-5.4 Av. 5 37	4.6 +	4.6 +	4.7 +	7.5 —	7.5 —	4.8 +	7.5 —	0.40-0.75 —	5.2-6.2 +	0.0-0 42 —	—	Streptococcus kefir
	1	Chains of 2-6 usually	Gamma	—	Acid not coagulated	Slight acid	. .	5.0	4 4 +	4.4 +	4 4 +	4.5 +	7.5 —	4.4 +	7.5 —	0.70 —	5.6 +	0.28 —	—	

TABLE 5

CULTURAL CHARACTERISTICS OF STREPTOCOCCI FROM MILK WITH AN ACIDITY OF 0.6% AND ABOVE

Group	Number of Cultures	Morphology	Hemolysis	Methylene Blue Reduced	Litmus Milk Reaction		Janus Green Reduced	P_H in Medium with Surface Tension of 42.8 Dynes	Final P_H in Fermentations							C c. of CO_2 from		NH_3—N from Peptone, Mg. per 100 C c. Excess over Control	Sodium Hippurate Hydrolyzed	
					30 C.	10 C.			Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin	Peptone	Dextrose			
1	51	Chains of 2-6 usually	Gamma some green	+	Typical	Typical	+	Range 4.6-5.0 Av. 4.84	4.3 +	4.3 +	7.6 —	4.8 +	4.8 +	7.6 —	7.6 —	Range 2.05-5.36 +	Range ..... —	Range 14 86-22.42 +	+	Streptococcus lactis Var. A
2	20	Chains of 2-6 usually	Gamma some green	+	Typical	Typical	+	4.6-5.0 Av. 4.86	4.3 +	4.3 +	7.6 —	4.8 +	4.8 +	7.6 —	7.6 —	2.94-3.55 +	..... —	7.57-21.72 +	+	
3	8	Chains of 2-6 usually	Gamma some green	+	Typical	Typical	+	4.7-4.9 Av. 4.77	4.3 +	4.3 +	4.3 +	4.8 +	4.8 +	7 6 —	7.6 —	2.64-3.90 +	..... —	20.18-22.0 +	+	
9	4	Chains of 2-6 usually	Gamma	+	Typical	Typical	+	4.7-5.0 Av. 4.87	4.3 +	4.3 +	4.3 +	4.7 +	7.6 —	7.6 —	7.6 —	3.20-5.80 +	..... —	20.74-21.86 +	+	
	3	Chains of 2-6 usually	Gamma	—	Acid not coagulated	Slightly acid	..	5.4	4.3 +	4.3 +	4.6 +	7.5 —	7.5 —	4.6 +	7.5 —	0.24-0.44 —	4.9-7.9 +	0.0-0.56 —	—	Streptococcus kefir
	2	Chains of 2-6 usually	Gamma	—	Acid not coagulated	Slightly acid	..	5.2	4.3 +	4.3 +	4.3 +	4.6 +	7.5 —	4.5 +	7.5 —	0.00-0 69 —	5.1-5.4 +	0.28 —	—	
	1	Chains of 2-6 usually	Gamma	—	Acid not coagulated	Slightly acid	..	5.4	4.4 +	4.6 +	4.4 +	7.5 —	7.5 —	7.5 —	7.7 —	0.49 —	5.5 +	0.28 —	—	

At this point it may be well to mention that numerous lactic streptococci have been isolated from a number of starters and have been found to fall into groups 1 and 5. They represent, therefore, both varieties A and B of Streptococcus lactis. Commercial starters were found more often to contain the strain of variety A which ferments salicin and mannite, but not saccharose. This is the strain most common in the normal sour milk we have studied.

The Streptococci of Milk with Acidities Ranging from 0.3 to 0.49%.—It will be remembered that in milk with an acidity of from 0.18 to 0.25%, Streptococcus kefir was found to be the predominant streptococcus. It may be seen from table 4 that in milk of higher acidity about 59% of the streptococci found were Streptococcus lactis. The 22 cultures were isolated from 8 composite samples of milk. Both varieties A and B of the organism were isolated, and the predominant strain was one most prevalent in milk of lower acidity.

The Streptococci of Milk with an Acidity of 0.6% and over.—A study of the streptococci from another series of samples of market milk, allowed to develop an acidity of 0.6% or more of acid, showed 92.3% of Streptococcus lactis and 6.7% of Streptococcus kefir, as will be seen from table 5. The 89 cultures were isolated from 29 composite samples of milk. Here again the strain predominating—the lactic streptococcus—in milk of lower acidities held the same position in sour milk. In all our collection of cultures from sour milk of different acidities in the nonsaccharose fermenting strains have been more prevalent than those capable of fermenting this sugar, and var. A was more common than var. B.

TABLE 6

PERCENTAGE OF DIFFERENT STREPTOCOCCI FOUND IN SOURING MILK

	Percentage of Acidity as Lactic Acid		
	0.18–0.25	0.30–0.49	0.60+
Streptococcus lactis	27.7	59.0	92.3
Streptococcus kefir	67.4	41.0	6.7
Miscellaneous streptococci	4.9	0	0

In table 6 is shown the percentage of different streptococci found in milk of different acidities. The figures give a clear picture of the majority streptococcus flora of souring milk. In addition it might be said that among the miscellaneous streptococci, none was found to be related to the streptococci of the udder, mouth or feces of cows. Such

organisms might have been present but not in sufficiently large numbers to be isolated at the dilution required to obtain the most prevalent types.

SUMMARY AND CONCLUSIONS

The predominating streptococcus flora of souring milk has been studied. Samples of the ordinary grade of market milk were collected and allowed to stand at about room temperature until different degrees of acidity were reached.

The conclusions are based on a study of 101 cultures from 40 composite samples of milk from 40 farms, having an acidity of from 0.18 to 0.25%, 22 cultures from 8 farms having an acidity from 0.30 to 0.49%, and 89 cultures from 22 samples of milk having an acidity of 0.6% and above.

Streptococcus kefir predominated in milk of low acidity and Streptococcus lactis in milk of high acidity. Neither of these organisms were in the udder, feces or mouth of cows, as is shown in other papers in this series. Not any of the streptococci found in the udder, feces or mouth of cows were isolated from souring milk. It appears, therefore, that Streptococcus kefir and Streptococcus lactis gain entrance into milk in small numbers and then develop rapidly so that overgrowth of other streptococci occurs.

Streptococcus kefir, by the ordinary cultural tests, appears to be like Streptococcus bovis, the typical streptococcus of cow feces. However, it is characterized by the production of CO_2 from dextrose and can, therefore, be readily distinguished from the cow fecal streptococcus.

Streptococcus lactis is characterized by the positive correlation between the quick reduction of litmus or the reduction of Janus green, or ammonium molybdate; growth at 10 C.; and the ability to reach a low final P_H in a special medium in which the surface tension has been reduced to 42.8 dynes by sodium glycocholate. The rate of acid formation is not particularly important, although cultures freshly isolated from milk produce acid rapidly and coagulate milk within 24 hours at 30 C. There appear to be two varieties, A and B of Streptococcus lactis, the first of which produces CO_2 and NH_3 from peptone, while the second does not. This ability to produce CO_2 and NH_3 seems to be correlated with a greater ability to reach a lower final P_H in the lowered surface tension medium. Var. A according to our results appears to be most prevalent. In commercial starters both

varieties have been found; some have var. A, others var. B. Several strains of each variety were found; the most common being the one which ferments dextrose, lactose, salicin, and mannite, but not saccharose. Some strains ferment dextrose and lactose, and others vary in combinations up to the fermentation of 5 test substances.

RELATION OF STREPTOCOCCUS FECALIS TO STREPTOCOCCUS LACTIS

STUDIES OF THE STREPTOCOCCI. X

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Some authors believe that the fecal streptococci are nothing more than lactic acid types. Kruse,[1] as early as 1903, stated that Streptococcus enteritis (Hirsh-Libman) and Enterococcus (Thiercelin) were nothing more than pure lactic acid bacteria. A similar belief was expressed by Sittler,[2] who felt that Enterococcus was closely related to Streptococcus acidi lactici (Lehmann-Neuman), now known as Streptococcus lacticus (Kruse) or Streptococcus lactis, in form, growth, and chemical activities, if not identical with it.

Various names are given to fecal streptococci in different countries. Thiercelin[3] found in the intestine a gram-positive streptococcus which he termed Enterococcus. This is the name used extensively by French writers. To what is apparently the same species the name Streptococcus fecalis was given by Andrewes and Horder.[4] The name Streptococcus enteritis was also given by Libman[5] to a fecal organism found by Hirsh.[6] It was the opinion of Tissier,[7] however, that Streptococcus enteritidis was different from Enterococcus, and this has been confirmed by the work of Dible.[8] According to this author, Streptococcus enteritis comprises a large group of true chain-forming streptococci of the feces, many of which are probably surviving salivary types, while the Streptococcus fecalis of Andrewes and Horder comprises the group of the Enterococcus.

It seems, therefore, that there may be two distinct species of fecal streptococci, the most common of which are Streptococcus fecalis or Enterococcus, and Streptococcus enteritis.

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[1] Centralbl. f. Bakteriol., I, O., 1903, 34, p. 737.
[2] Ibid., 1908, 47, p. 145.
[3] Compt. rend. Soc. de biol., 1899, 1, p. 269.
[4] Lancet, 1906, 171, p. 708, 775, 852.
[5] Centralbl. f. Bakteriol., 1897, 22, p. 376.
[6] Ibid., p. 369.
[7] Recherches sur la flore intestinale des nourrissons. Thesis, 1900.
[8] Jour. Path. & Bacteriol., 1921, 24, p. 3.

Although it has been suggested at least twice that the Enterococcus is identical with or similar to Streptococcus lactis, it appears that no great attention has been paid to these beliefs. If it is true, it is a matter of considerable importance. Perhaps the lack of specific data relative to Streptococcus lactis has caused this neglect, because the relationship could be easily overlooked unless one has before him the characteristics of both groups of streptococci.

While studying the lactic streptococci, the excellent paper by Dible [8] came to our attention, and we were impressed by the similarity of the reactions of the fecal streptococci to those of the cultures in our collection of lactic streptococci. Not only were the fermentation tests similar, but also hemolysis and heat resistance. We then decided to isolate a few fecal streptococci and study their reactions on the same basis as the lactic organisms.

CULTURAL CHARACTERISTICS

Samples of feces were collected on sterile swabs from 5 different persons. Blood-agar plates were made directly from 3 of the fecal samples. From these 3 samples some of the fecal material was inoculated into sterile milk, which was then heated to 60 C., held 15 minutes, and then incubated at 30 C. until it was curdled. It was then plated on blood agar, and cultures were isolated. This heating process is similar to the method used by Dible.[8] Streptococci were isloated by the heating method from one other sample, while the remaining sample was inoculated into milk which was then incubated for 48 hours at 30 C. without heating. When the milk was coagulated, blood agar plates were made and streptococci isolated.

By these methods of heating and incubation it was felt that we could isolate the same types found by Dible, and by direct plating obtain the majority streptococcus flora of human feces.

Thirty-three cultures were isolated, purified, and studied according to the methods previously described by Ayers and Mudge.[9] The medium for fermentation tests was somewhat different so far as infusion broth with 1% Parke-Davis peptone and 0.5% test substance was used. The reaction was adjusted to P_H 7.5 before sterilization.

The Janus green test is described in a previous paper [10] of this series on the streptococci of souring milk. The final P_H in a medium with lowered surface tension and its value as a means of helping to classify streptococci are shown in the same paper. More complete work on the

[9] Jour. Infect. Dis., 1922, 31, p. 40.
[10] Ayers, S. H.; Johnson, W. T., Jr., and Mudge, C. S.: Ibid.

influence of surface tension on the growth of streptococci may be found in another paper of this series.[11] We are indebted to Dr. Rupp of these laboratories for making the ammonia and hippurate tests.

The human fecal cultures have been arranged in 4 groups in table 1 for convenience in discussing them, and the cultures in each group separated according to their method of isolation. This has been done so as to show that the cultures are representative of the fecal flora.

Included in the table are the cultural characteristics of 3 strains of Streptoeoccus lactis var. A which have been described more completely in an earlier paper of this series.[10]

A study of table 1 reveals the striking resemblance between groups 1, 2, and 3 of the human fecal streptococci and the strains of Streptococcus lactis under groups 1A, 2A, and 3A. As was pointed out in the earlier paper, Streptococcus lactis is characterized by a positive correlation between a rapid and powerful reduction (as shown by litmus or Janus green), growth at 10 C., and the ability to reach a low final P_H in a medium, the surface tension of which had been reduced by sodium glycocholate to about 42.7 dynes.

The first 3 groups of the human fecal streptococci answer this requirement as a rule, although slightly less vigor in general was observed among the cultures. In litmus milk, for example, the typical reduction of litmus was usually observed in 24 hours, but the cultures did not produce acid so actively as the lactic streptococci. Neither did the fecal cultures reach quite so low a final P_H in the surface tension medium or in the usual fermentation tests.

The morphology of the human fecal cultures in groups 1, 2, and 3 was identical with that of Streptococcus lactis, and their colonies appeared the same on blood-agar plates. Both the human fecal and the lactic streptococci were heat resistant according to Dible's test.

From the cultural characteristics of the human fecal streptococci, which because of the methods of isolation probably represent the majority streptococcus flora, it appears that they are closely related to, if not identical with, Streptococcus lactis.

One culture, which constitutes group 4 of the human fecal cultures, appeared to be different from the rest. Long chains, if formed, did not grow at 10 C. and reached only P_H 6.8 in the surface tension medium. From the reaction of this culture we believe it to be a surviving mouth type, as it agreed closely with the human mouth culture in our collection.

[11] Ayers, S. H.; Rupp, P., and Johnson, W. T., Jr.: Ibid., 1923, 33, p. 202.

Comparison of Cultural Characteristics of Human Fecal Streptococci and Streptococcus Lactis

Group Number	Number of Cultures	Method of Isolation	Morphology	Hemolysis	Survive Heating at 60 C. for 30 Minutes	Litmus Milk Reaction		Janus Green Reduction	Final P_H in Medium with Surface Tension Lowered to 42.8 Dynes	P_H in Fermentation							CO_2 from Peptone	NH_3 Produced from Peptone	Sodium Hippurate Hydrolized	
						30 C. 24 Hours	10 C. 7 Days			Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin				
1	2	Incubation in milk	Oval shape Chains of 2-6 usually	Gamma green sometimes weak alpha	+	Typical	Growth +	+ —	Average 5.0	4.6 +	4.6 +	4.5 +	4.7 +	4.7 +	7.0 —	7.1 —	+	+	+	Human fecal Streptococci
	11	By heating	do.	do.	+	6 typical; not coag. 5 acid (1)	do.	8+ 3—	4.8-5.3 Average 5.1	do.	do.	do.	do.	do.	do.	do.	+	+	6+ 5—	
	9	Direct plating	do.	do.	+	Typical not coag.	do.	+	5.0-5.4 Average 5.2	do.	do.	do.	do.	do.	do.	do.	+	+	1+ 8—	
2	4	By heating	do.	do.	+	do.	do.	+	4.8-5.1 Average 5.0	4.6 +	4.6 +	7.1 —	4.7 +	7.1 —	7.0 —	7.2 —	+	+	+	
	4	Direct plating	do.	do.	+	2 do. 2 acid (2)	do.	+	5.0-5.1	do.	do.	do.	do.	do.	do.	do.	+	+	3+ 1—	
3	1	By heating	do.	do.	+	Acid	do.	+	5.0	4.6 +	4.7 +	7.2 —	4.8 +	4.9 +	7.3	7.2	+	+	0	
	1	Direct plating	do.	do.	+	Typical not coag.	do.	+	5.2	4.6 +	4.7 +	6.8 —	4.7 +	4.8 +	6.9 —	7.0 —	+	+	+	
4	1	Direct plating	Long chains	do.	+	Acid	—	+	6.8	4.6 +	4.6 +	4.7 +	5.5 +	7.1 —	7.1 —	7.1 —	+	+	0	
1A	—	—	Oval shape chains of 2-6	Gamma green or weak alpha	+	Typical	Growth +	+	4.5	4.3 +	4.3 +	4.3 +	4.6 +	4.5 +	7.6 —	7.6 —	+	+	+	Streptococcus lactis var. A
2A	—	—	do.	do.	+	do.	do.	+	4.6	4.3 +	4.3 +	7.6 —	4.8 +	7.0 —	7.6 +	7.6 —	+	+	+	
3A	—	—	do.	do.	+	do.	do.	+	4.7	4.3 +	4.3 +	7.6 —	4.8 +	4.8 +	7.6 —	7.6 —	+	+	+	

(1) Three of 5 cultures showed typical reduction of litmus when incubated at 37 C.
(2) One culture showed typical reduction of litmus when incubated at 37 C.

DISCUSSION OF RESULTS

The most prevalent strain of the human fecal cultures agreed closely with a strain of Streptococcus lactis found in souring milk when the acidity was low. The saccharose negative fecal cultures, groups 2 and 3, agreed well with the most prevalent strains of Streptococcus lactis found in sour milk.

After an extensive study of the Streptococcus lactis type and a study of a few human fecal cultures, we cannot help feeling that Streptococcus lactis is either closely related to Streptococcus fecalis or identical with it. It is true that the fecal cultures lack the vigor of the lactic cultures, but this can be easily explained by changes in environment. Probably every one consumes lactic streptococci in milk or dairy products, and since Streptococcus lactis can withstand the action of bile and low-surface tension, there is no good reason why it should not be found in the intestine. In the new environment it may grow, and its physiologic characteristics may be slightly modified.

When excreted from the intestine it is possible that these streptococci, in their passage to and through the soil, become subjected to conditions such that only the most vigorous can survive and that some of these find their way from the soil through various conveyors, as cattle feed, water, and air into milk containers in small numbers. In this manner they may contaminate milk.

What has been said represents merely a chain of possibilities and is not supported by data. But we do know that Streptococcus lactis is not recognized as being widely distributed, and yet it constantly gains entrance into milk in small numbers and then rapidly develops so as to overgrow other bacteria.

SUMMARY

A study has been made of 33 human fecal cultures. The predominant species, according to our present classification, is termed Streptococcus fecalis or Enterococcus.

It appears that Streptococcus fecalis is similar to, if not identical with, Streptococcus lactis. Earlier investigators observed the resemblance, and we have found they agree closely in what we believe to be fundamental physiologic reactions.

We hesitate to say that Streptococcus fecalis is identical with Streptococcus lactis, without further study, but we hope that the facts presented will stimulate other investigators to take up this interesting question.

INOCULATION OF WHITE MICE WITH PFEIFFER'S BACILLUS: MIXED CULTURES

INFLUENZA STUDIES. XV *

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Experiments on the effects of simultaneous inoculation of white mice with Pfeiffer's bacillus and some of the gram-positive cocci have been reported by me.[1] The object of the experiments now reported is to test the finding of increased virulence and to attempt to throw some light on the mechanism of the symbiotic relations of these bacteria.

The experiments of Jacobson,[2] Roos,[3] Yanagisawa,[4] Albert and Kelman,[5] and Wolf[6] have shown that the simultaneous injection of Pfeiffer's bacillus with other bacteria raises the virulence of the Pfeiffer bacillus. Yanagisawa[4] measured the increase and showed that the other bacteria (Streptococcus, Staphylococcus, Dipl. pneumoniae, Dipl. catarrhalis) injected with the Pfeiffer bacillus were relatively more influenced by the symbiotic relation. Albert and Kelman[5] concluded that the injection of the Pfeiffer bacillus favors the invasion of tissue by other bacteria, as well as conversely.

Wassermann and Keysser,[7] writing on mixed and secondary infection, refer repeatedly to clinical and laboratory findings of Pfeiffer's bacillus as having important pathologic significance because of relations with other bacteria, especially the pneumococcus. Such observations, combined with the experimental evidence and the suspected relation of this organism to influenza, have led some investigators to attempt to produce influenza in man by inoculating volunteers with mixed cultures. In 1918-1919, Rosenau, McCoy and others,[8] made a thorough study of the possibilities of mixed cultures in producing this disease. The material came from influenza patients and was handled in every conceivable manner, even with the addition of known cultures, but with a few possible exceptions, the results were negative. Yamanouchi, Sakakani and Iwashima[9] were not able to reproduce influenza in 14 supposedly susceptible persons by inoculating them either with pure cultures of the Pfeiffer bacillus or with this organism mixed with cocci and other bacteria usually found in the sputum of influenza patients. On the other hand, Huntoon and Hannum[10] report that

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[1] Jour. Infect. Dis., 1922, 30, p. 433.

[2] Arch. de Med. Exper., 1901, 13, p. 425.

[3] Jour. Immunol., 1919, 4, p. 189.

[4] Kitasato Arch. Exper. Med., 1919, 3, p. 85.

[5] Jour. Infect. Dis., 1919, 25, p. 433.

[6] Centralbl. f. Bakteriol., I, O., 1920, 84, p. 241.

[7] Kolle und Wassermann, Handbuch der Pathogenen Mikroorganismen, 1912, 1, p. 632.

[8] Hygienic Laboratory Bull. 123, 1921.

[9] Lancet, 1919, 196, p. 971.

[10] Jour. Immunol., 1919, 4, p. 167.

guinea-pigs inoculated with Pfeiffer's bacillus or its extracts succumbed spontaneously and experimentally to subsequent exposures to and inoculations with various organisms, with the appearance of secondary lesions.

It thus appears to be established that the virulence of the Pfeiffer bacillus and other bacteria is enhanced by experimental association in the animal body, but such association is probably not capable of producing typical symptoms of influenza in man.

The technic now used was different from that in my first series, in that broth cultures of the cocci were used instead of suspensions from blood agar slants. This allowed for greater precision in measuring the amounts injected. Otherwise the technic was as follows: the Pfeiffer bacillus was grown for 23-25 hours on chocolate agar slants of standard size as previously described. Emulsions were made in sterile salt solution just prior to inoculation. The purity of these cultures and of the gram-positive cocci was controlled by staining before inoculation. Injections were made in triplicate usually, sometimes in duplicate.

The first set of experiments was conducted 10 to 12 months after those already reported and the same strain of Pfeiffer's bacillus (No. 56) was used with stock cultures of Strep. hemolyticus, Strep. viridans, and the pneumococcus (type I). The strains of streptococci in pure culture did not kill in the amounts injected (up to 1 c c of blood broth culture, 24 hours' incubation), nor were simultaneous injections with ⅛ slant of Pfeiffer's bacillus fatal. These avirulent stock strains of the cocci were used to determine whether their virulence could be raised by association with ⅛ slant of an old strain of Pfeiffer's bacillus. Such was not the case.

When the same tests were repeated with the substitution of a stock strain of pneumococcus (type I) for the streptococci, both the control mice inoculated with the pure culture of the coccus and the mice inoculated with mixed cultures died. The addition of the bacillus did not hasten the death of the mice appreciably. The bacillus, together with the pneumococcus, was isolated from the heart blood of only a few mice. Apparently a strain of Pfeiffer's bacillus long cultivated on artificial medium was not sufficiently raised in virulence by mixture with gram-positive cocci to take a part in killing the mice injected; nor was the virulence of the cocci noticeably enhanced by such association.

The next set of experiments was a repetition, except for the use of freshly isolated strains of Pfeiffer's bacillus and Strep. hemolyticus. The new strain of Pfeiffer's bacillus (AFR.) was recovered from the nasopharynx of a normal person. Two strains of Strep. hemolyticus were used: S1 from a sore throat, and HG from a case of pneumonia of which it was the causative organism. Another stock strain of the pneumococcus (type I), which had been cultivated for an indeterminable length of time, was employed.

The minimal lethal dose of the Pfeiffer bacillus was ¼ slant; 12 of 15 mice recovered when inoculated with ⅛ slant; and 30 of 31 mice recovered when inoculated with 1/16 slant. This last amount (1/16 slant) was chosen as a distinctly sublethal dose and was used as a constant factor in all simultaneous and alternating injections to be reported. The cocci were injected in varying amounts of a 24-hour broth culture, trying thus to find the sublethal range. Control injections of the cocci were made with each experiment using material for this purpose from the same culture as for the mixed injections. In this way, each experiment was complete in itself.

When mice died, the peritoneal fluid and heart blood were cultivated on chocolate agar plates. The organisms so isolated were identified by characteristics of the colonies and microscopically by the Gram reaction, morphology and

arrangement. The periods of survival fell distinctly into 2 groups and as such are reported in the tables, together with the total hours of survival. The first group died within 48 hours ("early deaths"), and the second group after that time ("delayed deaths"). In the following discussion only the totals are presented, but it should be noted that the number of "delayed deaths" was nearly the same throughout an experiment, making the "early deaths" the significant and comparable results. The mice that died after 48 hours commonly gave a pure culture of the coccus in the heart blood and showed an abscess at the site of inoculation. Furthermore, these mice usually died after an apparent recovery, all of these facts indicating that, as a rule, the immediate results were due to the action of the mixed injections, while the delayed results were due to a second infection with the coccus at the site of injection.

The results of the simultaneous injections of the Pfeiffer bacillus and Strep. hemolyticus (2 strains) showed that the mixtures killed a higher proportion of mice in a shorter time than the controls. In 4 experiments, 77 mice were injected with streptococcus and Pfeiffer's bacillus, of which 72 died with an average period of survival of 35 hours. The bacillus was recovered from the heart blood of 50 and the streptococcus from 55 of these fatally injected mice. At the same time, 78 mice as controls were injected with the coccus alone, of which 48 died with an average period of survival of 56 hours. Some experiments showed more strikingly than others that the Pfeiffer bacillus with the streptococcus kills more frequently and more quickly than the streptococcus alone. Thus sublethal doses of the streptococcus with the bacillus were often fatal (table 1).

When Pfeiffer's bacillus was injected into mice together with the pneumococcus (stock strain, type I), the results were not so striking since it was my experience that although the dilutions of the broth culture of the pneumococcus was at times as high as 1 : 50,000,000, I did not find a dosage that would not kill eventually. Hence, all the mice, whether inoculated with both organisms or with the pneumococcus alone, died. The average period of survival was reduced, in one experiment 31%, and it is perhaps significant that the bacillus was recovered from the heart blood of 26 of 41 mice fatally injected. In the case of the pneumococcus which is so virulent for white mice, the Pfeiffer bacillus did not seem to play an important rôle as a symbiotic organism (table 1).

It should be recalled that when lethal doses of this bacillus are injected in pure culture, it is difficult to recover it from the heart blood, and in such experiments [1] I was able to recover it in pure culture from 68%, and with a contaminating organism from 86%, of mice fatally injected. The findings following mixed injections should be compared with these figures to show their relative importance. The Pfeiffer bacillus was frequently isolated from the heart blood many hours after the mixed injection, and it seemed important to determine whether this persistence was due to the presence of the other organism, or whether the bacillus were present merely as a noncombatant. If its late appearance occurred only with the cocci, it would indicate some aggressive action, and the higher mortality following the mixed injections would be partly explained.

To approach this question, 28 mice were inoculated with 1/16 slant each, which is the dose used in all the experiments with this strain, AFR. Two mice were killed after varying lengths of time and cultures made from their heart blood and peritoneal fluid. The organism was found to invade the blood stream in 45 minutes and was regularly recovered from the heart blood up to 7 hours and once after 9 hours, but not later. It was recovered regularly from the peritoneal fluid up to 22 hours, and once after 34 hours, but not later. Following the mixed injections of this bacillus and these gram-positive cocci, the bacillus was recovered

TABLE 1

SUMMARY OF RESULTS OF SIMULTANEOUS AND ALTERNATING INJECTIONS OF PFEIFFER'S BACILLUS (A. F. R.) WITH GRAM POSITIVE COCCI

Organisms Injected with Pfeiffer's Bacillus	Pfeiffer's Bacillus Followed by the Gram-Positive Coccus			Gram-Positive Coccus Followed by Pfeiffer's Bacillus			Simultaneous Injection			Control Injections: Gram-Positive Coccus	
	No. of Mice Injected	No. and Percentage That Died or Recovered	Pfeiffer's Bacillus in Heart Blood	No. of Mice Injected	No. and Percentage That Died or Recovered	Pfeiffer's Bacillus in Heart Blood	No. of Mice Injected	No. and Percentage That Died or Recovered	Pfeiffer's Bacillus in Heart Blood	No. of Mice Injected	No. and Percentage That Died or Recovered
Strept. hemolyticus, Sl.	..	.....	..	..	.....	..	18	.....	..	18	.....
Early deaths	..	.....	..	..	.....	..	..	18 (100%)	13	..	12 (60%)
Delayed deaths	..	.....	.	..	.....	..	..	0	.	..	3 (17%)
Total	..	.....	..	..	.....	..	..	18 (100%)	13	..	15 (83%)
Recovered	..	.....	..	..	.....	..	..	0	..	..	3 (16%)
Strept. hemolyticus, HG											
Exper. 1	..	.....	..	..	.....	..	18	.....	..	18	.....
Early deaths	..	.....	..	..	.....	..	..	14 (78%)	7	..	7 (39%)
Delayed deaths	..	.....	..	..	.....	..	..	4 (22%)	1	..	5 (28%)
Total	..	.....	..	..	.....	..	..	18 (100%)	8	..	12 (66%)
Recovered	..	.....	..	..	.....	..	..	0	..	..	6 (33%)
(Same)—Exper. 2	21	.....	..	21	.....	..	18	.....	..	21	.....
Early deaths	..	16 (76%)	12	..	12 (57%)	10	..	15 (83%)	14	..	9 (43%)
Delayed deaths	..	4 (19%)	0	..	5 (24%)	1	..	2 (11%)	0	..	7 (33%)
Total	..	20 (95%)	12	..	17 (81%)	11	..	17 (94%)	14	..	16 (76%)
Recovered	..	1 (5%)	..	..	4 (19%)	..	..	1 (6%)	..	..	5 (24%)
(Same)—Exper. 3	21	.....	..	22	.....	..	23	.....	..	21	.....
Early deaths	..	13 (62%)	13	..	9 (41%)	8	..	15 (65%)	15	..	2 (10%)
Delayed deaths	..	3 (14%)	1	..	3 (15%)	0	..	4 (17%)	0	..	3 (14%)
Total	..	16 (76%)	14	..	12 (55%)	8	..	19 (82%)	15	..	5 (24%)
Recovered	..	5 (24%)	..	..	10 (45%)	..	..	4 (18%)	..	..	16 (76%)
Pneumococcus Type 1											
Exper. 1	..	.....	..	..	.....	..	18	.....	..	19	.....
Total deaths	..	.....	..	..	.....	..	..	18 (100%)	12	..	19 (100%)
Recovered	..	.....	..	..	.....	..	..	0	..	..	0
(Same)—Exper. 2	23	.....	..	23	.....	..	23	.....	..	23	.....
Total deaths	..	23 (100%)	12	..	23 (100%)	5	..	23 (100%)	14	..	23 (100%)
Average hours of survival	..	(21)	..	..	(21)	..	..	(18)	..	..	(26)
Recovered	..	0	..	..	0	..	..	0	..	..	0

Early deaths, within 48 hours; delayed deaths, after 48 hours.

regularly up to 29 hours. It was cultivated from the peritoneal cavity up to 40-50 hours. It can be said definitely that the Pfeiffer bacillus invades the blood stream of white mice even in sublethal doses; that there seems to be a multiplication under these conditions; that the effects are probably due to a septicemia and not only an intoxication from the original inoculum remaining in the peritoneal cavity; that this proliferation is more apparent when the organism is inoculated with these gram-positive cocci; and that it takes an active part in bringing about the result of mixed injections, which is a more certain and rapid death.

Further experiments with Pfeiffer's bacillus (AFR) and Strep. hemolyticus (HG) and the pneumococcus were conducted in an attempt to determine which of the organisms injected played the more active part. First, Pfeiffer's bacillus and Strep. hemolyticus were injected alternately, and second, immunized mice were used to determine whether the effects on one organism could be neutralized, leaving the other one to exert its effects alone. Suitable controls were run from the same culture of the coccus.

A period of 2 hours between the injection of the Pfeiffer bacillus and the coccus was chosen, because it had been determined that with the dose used (1/16 slant) this organism invaded the blood stream in less than 2 hours. If it exerted a specific action on the mouse, it might do so in that time. In the converse experiments, the coccus was injected 2 hours before the bacillus. From the same cultures of the coccus and bacillus identical amounts were injected simultaneously, and as controls the coccus was injected alone.

Such a series of tests, when conducted with Pfeiffer's bacillus and Streptococcus hemolyticus (HG), disclosed the fact that the mice inoculated first with the bacillus followed by the streptococcus died with about the same proportion of fatalities and about as quickly as those mice that were inoculated simultaneously. Following these groups came the mice inoculated first with the coccus then with the bacillus, and finally the controls.

This series of tests was repeated with similar results (table 2) and taking into consideration the range of dosage of the streptococcus, the numbers of mice used, and the finding of the bacillus in the heart blood of 74 of the 101 mice fatally injected with the 2 organisms (including both "early" and "delayed deaths"), the following conclusions can be drawn: The virulence of both organisms was probably increased; if the bacillus invaded the blood stream with or before the streptococcus, it acted as a kind of sensitizer for the second organism, aiding its invasion perhaps, and encouraging more certain and rapid results; in the amount used, the bacillus may have been more important as a primary invader than the streptococcus. Or it may be that the streptococcus when injected first caused the host to summon its defensive powers so that the combined action of the two organisms did not obtain.

Similar tests with the pneumococcus did not disclose such differences among the groups injected alternately. All mice were killed: first those injected simultaneously (averaging 18 hours of survival), followed by those groups injected with alternating doses (both 21 hours), finally the controls injected with the pneumococcus alone (26 hours). Although the mixed injections killed sooner, the order of injection made little difference. It should be noted, however, that among the 23 mice injected with Pfeiffer's bacillus first, 12 furnished the bacillus in the heart blood; 14 of the same number of mice injected simultaneously yielded the bacillus in the heart blood, while it was recovered from only 5 mice injected with the pneumococcus first. Apparently the pneumococcus killed certainly and rapidly of itself, and when the Pfeiffer bacillus was injected at the same time or before this coccus, it took a part in the invasion, but the time of survival in any event was so short that it could not demonstrate its activity. When the pneumococcus

was injected first, it appears that either the defenses of the host were aroused or else that the bacillus as a secondary invader was as unimportant as it seemed to be with the streptococcus. It is possible that had a freshly isolated strain of the pneumococcus been used, a more definite symbiotic relation with Pfeiffer's bacillus might have been demonstrated.

TABLE 2

ALTERNATING AND SIMULTANEOUS INJECTIONS OF PFEIFFER'S BACILLUS (A. F. R.) AND STREPTOCOCCUS HEMOLYTICUS (H. G.) INTO NORMAL AND IMMUNIZED MICE (EXPER. 3)

Order of Injection	Number Injected	Number Recovered	Number Died		Average Period of Survival in Hours		Pfeiffer's Bacillus in			
							Heart Blood		Peritoneal Fluid	
			Early and Delayed	Total	Early and Delayed	Total	Early and Delayed	Total	Early and Delayed	Total
Pfeiffer's bacillus followed by Strep. hemolyticus (a) Normal mice	21	5	E 13 D 3	16	E 14 D 88	28	E 13 D 1	14	E 13 D 2	15
(b) Mice immunized against Pfeiffer's bacillus	8	7	D 1	1	D 165	165	0		0	
Strep. hemolyticus followed by Pfeiffer's bacillus (a) Normal mice	22	10	E 9 D 3	12	E 22 D 158	49	E 8 D 0	8	E 9 D 2	11
(b) Mice immunized against Pfeiffer's bacillus	8	1	E 4 D 3	7	E 30 D 212	108	E 3 D 0	3	E 4 D 1	5
Simultaneous injections (a) Normal mice	23	4	E 15 D 4	19	E 16 D 163	47	E 15 D 0	15	E 15 D 0	15
(b) Mice immunized against Pfeiffer's bacillus	8	1	E 5 D 2	7	E 22 D 150	59	E 3 D 0	3	E 4 D 1	5
(c) Mice immunized against Strept. viridans	7	0	E 6 D 1	7	E 11 D 68	19	E 6 D 1	7	E 6 E 1	7
Controls injection of Strept. hemolyticus (a) Normal mice	21	16	E 2 D 3	5	E 21 D 140	92				
(b) Mice immunized against Pfeiffer's bacillus	8	6	E 2 D 0	2	E 15	15				
(c) Mice immunized against Strept. viridans	7	1	E 4 D 2	6	E 18 D 198	78				

Amounts injected in duplicate or triplicate: Pfeiffer's bacillus, 1/16 standardized chocolate agar slant; Strept. hemolyticus, 1 c.c. to 0.0001 c.c. blood broth culture; E, early death, within 48 hours; D, delayed death, after 48 hours.

The last set of experiments was with Strep. hemolyticus (HG) and Pfeiffer's bacillus (AFR) and involved alternating and simultaneous injections. At the same time, parallel injections were made into mice that had been immunized by repeated sublethal then lethal doses of Pfeiffer's bacillus (No. 56 and AFR). In the same manner, other mice were used that had been injected several times with

Strep. viridans. Because of the limited number of immunized mice, they had to be used sparingly. Hence they were placed in each group of mice and were injected in duplicate with the largest doses.

The inoculation of Pfeiffer's bacillus followed by Strep. hemolyticus again resulted in a high proportion of deaths, but identical injections into 8 mice immunized against Pfeiffer's bacillus resulted in but 1 fatality and that after 125 hours. The amounts used were the usual dose (1/16 slant) of the bacillus, followed by the largest doses (1 c c.-0.1 c c.) of the streptococcus in duplicate. However, when the order of injection was reversed, fewer normal mice died, but of the 8 immunized mice similarly injected, 4 died within 48 hours and 3 died after that period. When normal mice were injected with both organisms simultaneously, there was a high rate of mortality much like that of the group injected first with the bacillus. Immunized mice injected with the same doses suffered a high rate of mortality, 5 dying before 48 hours and 2 later. Finally, of 8 immunized mice injected with the streptococcus alone with the same amounts as throughout the experiment, only 2 died, which was close to the mortality of normal mice under the same conditions.

To control in some way the specificity of any action resulting from the immunization against Pfeiffer's bacillus, the mice immunized against Strep. viridans were inoculated simultaneously with Pfeiffer's bacillus and Strep. hemolyticus with doses identical with those given normal mice and mice immunized against Pfeiffer's bacillus. The mortality was high under both circumstances, and therefore it can be inferred that the results coming from the use of mice immune to Pfeiffer's bacillus were somewhat specific.

These experiments indicate that the high mortality resulting from the injection of Pfeiffer's bacillus before Strep. hemolyticus was due to the combination of organisms, since the action of Pfeiffer's bacillus early injected seemed to be offset in immunized mice, perhaps by lysis of the organism or neutralization of the toxic substances. These immune mice then reacted as normal mice to streptococcus alone. This would seem to indicate that when the bacillus preceded the coccus in the order of injection, the mice immunized to Pfeiffer's bacillus were left to the action of the streptococcus alone. But when the bacillus and coccus were injected at the same time, this protective function could not be utilized sufficiently to overcome the combined forces. This is further illustrated by the fact that when the streptococcus preceded the bacillus, the mortality was very much like that of the normal mice injected in the same manner. Apparently this neutralizing or protective function belonging to mice immune to the bacillus was specific for the bacillus since the susceptibility of the immune mice to Strep. hemolyticus alone was similar to that of normal mice.

Table 2 is a record of one of the experiments reported, and is chosen because it illustrates most of the points here discussed, especially those involving the use of immunized mice. The experiments on simultaneous and alternating injections are summarized in table 1.

DISCUSSION

The finding that a preliminary sublethal dose of Pfeiffer's bacillus was capable of making mice susceptible to sublethal doses of Strep. hemolyticus (and to a less extent, to the pneumococcus), when the reverse was not true, supports the experiments of Huntoon and Hannum [10] and others previously referred to, and goes farther to show that this effect on the host is specific in that it can be neutralized by immune

bodies. Such neutralization operated only when Pfeiffer's bacillus was injected ahead of the second invader, which seemed to allow the immune bodies to act.

A similar increase in susceptibility of the host to the Pfeiffer bacillus through the action of the gram-positive cocci was not demonstrated in these experiments. This may have been due to the technic employed, which consisted in using large doses of the cocci preceding small amounts of the bacillus. This objection hardly seems reasonable since when the same amounts were injected together, a symbiotic action resulted in which the Pfeiffer bacillus took an active part, as judged by the culture findings of the heart blood. It might also be said that possibly the Pfeiffer bacillus was more important as a primary than as secondary invader.

CONCLUSIONS

Stock strains of Pfeiffer's bacillus and Strep. hemolyticus, Strep. viridans and the pneumococcus carried on artificial medium for long periods of time (2-3 years) did not show increased invasive power on simultaneous injection into white mice. A freshly isolated strain of Pfeiffer's bacillus was found to be increased in virulence when injected (1/16 slant) with recently isolated strains of Strep. hemolyticus or with a stock strain of the pneumococcus, type I. The same strains of the streptococcus showed increased virulence with this bacillus, but this property of the pneumococcus was not so markedly increased.

The Pfeiffer bacillus was isolated from the heart blood of about 65% of mice dying after injection with 1/16 slant of this organism simultaneously with the pneumococcus and with Strep. hemolyticus. When this amount of the culture was injected alone, the bacillus was recoverable from mice killed at varying intervals up to 9 hours, from the heart blood, and up to 34 hours from the peritoneal fluid. Following fatal mixed inoculations, it was recovered from the heart blood regularly up to 20 hours, but also in some instances up to 35, 50 and even 78 hours, and from the peritoneal cavity regularly 40-50 hours after injection, but also 114, 160 and 250 hours.

When the Pfeiffer bacillus (1/16 slant) was injected 2 hours before or simultaneously with Strep. hemolyticus, a higher proportion of early mouse deaths occurred than when the streptococcus preceded the bacillus or was injected alone. A stock strain of the pneumococcus (type I) similarly injected did not show the same results so definitely, although the average period of survival was shorter in mixed than in pure culture inoculations.

Mice immunized against Pfeiffer's bacillus did not succumb to the injections of this organism followed by Strep. hemolyticus as regularly as normal mice. But their susceptibility either to the reverse order, simultaneous inoculations, or Strep. hemolyticus alone was similar to that of normal mice. Mice previously immunized to Strep. viridans reacted as normal mice to identical inoculations. In the doses used, Pfeiffer's bacillus seemed to act as a sensitizer of the host to Strep. hemolyticus, but the converse was not found to be true. Mice immunized to the Pfeiffer bacillus apparently neutralized this power of sensitization when the bacillus was injected before the second organism, such mice then reacting to the second organism alone.

COMPARATIVE STUDY OF GROWTH AND BIOCHEMICAL ACTIVITIES OF B. BOTULINUS, B. SPOROGENES AND B. TETANI, WITH NOTES ON CHEMICAL BEHAVIOR OF B. BOTULINUS TYPE[1] C*

STUDIES ON METABOLISM OF ANAEROBIC BACTERIA I

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

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In the course of an extensive study on anaerobic metabolism to be reported shortly, it was noted that the biochemical activities of various strains of B. botulinus, B. sporogenes and B. tetani presented definite similarities as well as slight differences. A review of the literature not only disclosed many contradictory experimental results, but failed to reveal agreement among various workers concerning the interpretation of similar findings regarding these 3 anaerobes. The reliable anaerobic plating method to be described by one of us (C. C. D.) offered an opportunity to estimate accurately the number of viable organisms participating in the metabolic activities. The work of Wilson[2] suggests that dilution with saline or even Ringer's solution introduces an appreciable error when young cultures are plated. It will be shown that the use of peptic digest-beef heart infusion-0.5% glucose agar as a carrier for the 7-10% sheep's blood provided a solid medium of unsurpassed biologic value for anaerobic growth. Its similarity to the liquid medium in which the 3 anaerobes were grown and to the peptic digest-beef heart infusion in which dilutions were made prior to plating, lessened any shock due to transfer.

Moreover, since it will be demonstrated that this counting method functions also as a means of detecting contaminations, its use guarded the intensive comparative study of the 3 organisms from any suspicion of impurity of cultures. Zeissler's[3] conclusions regarding the specificity

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[1] Bengtson: Pub. Health Rept., 1923, 38, p. 340.

[2] Jour. Bacteriol., 1922, 7, p. 406.

[3] Ztschr. f. Hyg. u. Infektionskrankh., 1918, 86, p. 52.

of colony form of anaerobes on blood-agar plates have been amply confirmed, and the dependable anaerobic conditions provided by previously described anaerobe jars [4] insured vigorous growth and consequent representative colony development. Photographs of typical plates illustrating the individual colony characteristics are included (fig. 1). The 3 anaerobes produce hemolytic zones surrounding the colonies, but B. sporogenes has much the largest zone of hemolysis, in the center of which the colony appears as a rather dense core from which filaments radiate into the hyaline area. The cores of B. tetani colonies are less

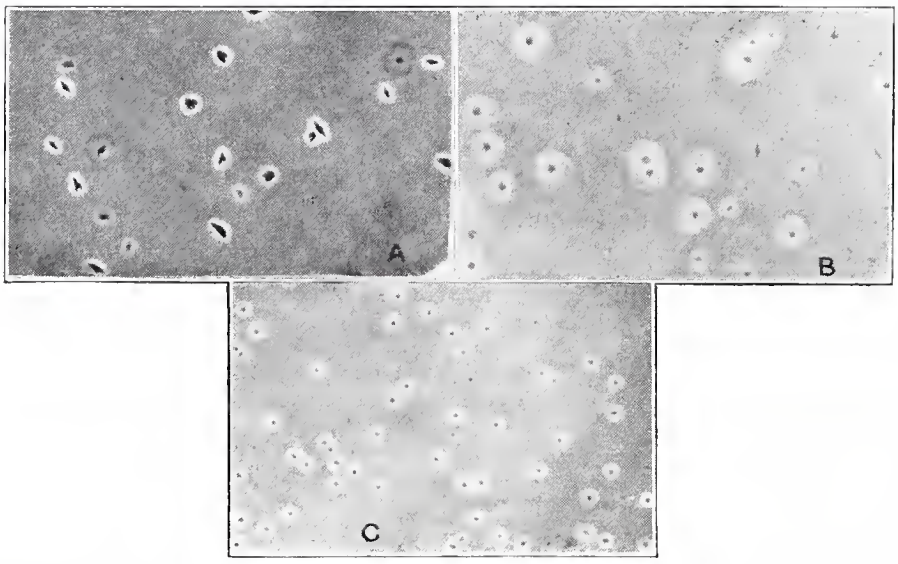

Fig. 1.—Appearance of colonies of B. botulinus (A), B. sporogenis (B) and B. tetani (C) on peptic digest beefheart-glucose-sheep blood agar.

dense than those of B. sporogenes, and the filamentous extensions into the hyaline area are shorter and more delicate. The coloring of the B. tetani colonies is characteristic. The dark nucleus is surrounded by a lighter zone, comprising approximately two-thirds of the hemolyzed area, which in turn is bordered by a narrower band of clear hemolysis. B. botulinus colonies, on the contrary, are clear cut and definite disks, usually lens shaped, surrounded by a clear, bacteria-free zone of hemolysis. The illustrations serve admirably to emphasize the specific characteristics of the 3 types of colonies.

[4] Jour. Infect. Dis., 1922, 31, p. 617.

REVIEW OF BIOCHEMICAL ACTIVITIES OF THE THREE ANAEROBES

Measures of Proteolysis.—Various studies of the chemical activities of anaerobes have yielded information of value. Heller,[5] basing her differentiation on P_H determinations after 20 days' incubation at 37 C., considered an alkaline reaction (P_H greater than 7) a dependable mark of proteolysis. Hall[6] has recently reviewed the means available for ascertaining the presence or absence of proteolytic activity. While he urges for purposes of classification the adoption of routine procedures which involve only the detection of destruction of proteins, he, it appears, considers identification of the products of protein breakdown the more reliable criterion. Kendall[7] has emphasized the need of the pathologist and general practitioner for carefully controlled chemical analyses of the products of bacterial action. Even more recently Braun and Cahn-Bronner[8] have ably set forth the advantages to be gained from studies of bacterial metabolism. The extensive biochemical work of the British investigators had its impetus in the quest for a rational treatment of anaerobic wound infections.

The 3 organisms under consideration generally have been classified as proteolytic, although not unanimously so. Von Hibler[9] placed the 3 in his most proteolytic group, as Zeissler[10] did B. tetani, but it may be mentioned that Heller[11] discounted these findings on the score of probable contamination with B. sporogenes. The British Medical Committee[12] included B. sporogenes and B. tetani in the proteolytic group, but placed B. botulinus in the group of organisms with "saccharolytic tendencies predominating." Henry[13] placed B. tetani with B. sporogenes in the proteolytic group, but emphasized the activity of the latter as vigorously proteolytic. Wolf and Harris[14] found B. sporogenes attacked proteins with great violence, and the former[15] reports high ammonia formation by B. tetani which he observes, "is typical of strongly proteolytic organisms, such as B. sporogenes and B. histolyticus." Heller[5] classes B. tetani among the less active

[5] Jour. Bacteriol., 1921, 6, p. 521.
[6] Jour. Infect. Dis., 1922, 30, p. 445.
[7] Endocrinology and Metabolism, 1922, 3, p. 663.
[8] Biochem. Ztschr., 1922, 131, p. 226.
[9] Untersuchungen über pathogene Anaerobien, 1908.
[10] Ztschr. f. Infektionskrankh. d. Haustiere, 1920, 21, p. 1.
[11] Jour. Infect. Dis., 1922, 30, p. 18.
[12] Medical Research Committee; Special Report Series, No. 12, 1917.
[13] Jour. Path. and Bacteriol., 1917, 21, p. 344.
[14] Ibid., p. 386.
[15] Brit. Jour. Exper. Path., 1921, 2, p. 266.

proteolytic anaerobes and B. botulinus only slightly less "highly proteolytic" than B. sporogenes. Hall [6] considers B. tetanti weakly proteolytic, B. botulinus predominantly proteolytic and B. sporogenes strongly proteolytic. Kahn [16] found the proteolytic activity of B. botulinus and B. sporogenes equally strong, while B. tetani he designated more "gradually proteolytic." Kendall, Day and Walker,[17] on the other hand, found B. botulinus and B. tetani "chemically relatively inert," and B. sporogenes "strongly proteolytic."

Significance of Protein Degradation Products.—Regarding the significance of the products of protein decomposition found in bacterial cultures, Kendall and Walker,[18] it is evident, formerly considered ammonia formation the most reliable index of the extent of proteolysis. In later work, however, Kendall, Cheetham, and Hamilton [19] apparently are ready to ascribe proteolytic activity to the work of exo-enzymes, and to consider the ammonia as a legitimate measure of intracellular or endogenous metabolism only. They have experimentally demonstrated the nonappearance of ammonia in mediums acted on by bacteria-free filtrates of proteus cultures. There was a rapid protein decomposition, but no ammonia formation in such mediums in contrast to a rapid accumulation of ammonia in mediums containing living proteus bacilli. Waksman [20] considered the study of ammonification of doubtful importance in revealing the proteolytic activities of telluric microorganisms. De Bord's [21] recent paper emphasizes the significance of amino acids as a measure of proteolysis under certain conditions, and at the same time he denies the proteolytic significance of ammonia. Itano [22] earlier determined the extent of proteolysis by measuring the amino acid increase, while Benton,[23] it would seem, placed not quite so much confidence in the significance of this product since she checked her amino acid determinations by simultaneous ones on the noncoagulable protein. Sears [24] is inclined to use an average of the amino acids and ammonia as the more reliable criterion. He believes neither the ammonia nor the amino acids present in culture mediums at any given time represents the amounts actually produced, both substances

[16] Jour. Med. Res., 1922, 43, p. 155.
[17] Jour. Infect. Dis., 1922, 30, p. 141.
[18] Jour. Biol. Chem., 1913, 15, p. 277.
[19] Jour. Infect. Dis., 1922, 30, p. 251.
[20] Jour. Bacteriol., 1918, 3, p. 475.
[21] Ibid., 1923, 8, p. 7.
[22] Mass. Agric. Exper. Station Bull., 167, 1916.
[23] Jour. Infect. Dis., 1919, 25, p. 231.
[24] Ibid., 1916, 19, p. 105.

being used in the synthesis of bacterial protein. As has just been noted, De Bord recognized this loss by synthesis, and Kendall and Bly [25] also noted a diminution of ammonia in B. coli cultures. Bierema [26] found that the utilization of inorganic nitrogren by various organisms depended on the presence of an appropriate carbohydrate. Doryland [27] takes an even more extreme view, for he writes regarding his experimental results with soil ammonifying bacteria: "If these organisms can assimilate as well as liberate ammonia, is there any proof that all the nitrogen from some or all ammonifiable material may not have been liberated as ammonia? The bacteria then assimilate sufficient to balance the limiting factor in the bacterial ration." Long,[28] working with tubercle bacilli, is inclined to such a view.

The conclusion of Ayers, Rupp and Mudge,[29] with regard to the part played by ammonia in bacterial metabolism, is unique. They found, "Our results seem to point to the probability that ammonia and carbon dioxide are derived in the degradation of some substance present in small amounts in some kinds of peptone."

OPTIMUM MEDIUM FOR GROWTH OF THE THREE ANAEROBES

In making comparisons of the biochemical activities of anaerobic organisms the advantages of using mediums of high biologic value as a means of allowing individual characteristics to express themselves fully was early emphasized by Smith, Brown, and Walker [30] and later by Heller [31] The superiority of mediums containing beef heart for the growth of anaerobes was recognized by Robertson [32] and by Holman.[33] That the biologic value of culture mediums is in the main dependent on the presence of easily available nitrogen was experimentally demonstrated by Bainbridge [34] and Rettger and his associates.[35] Among those whose pioneer contributions have resulted in the more general use of digest products may be mentioned Dalimier and Lancereaux [36] and Cole

[25] Ibid., 1922, 30, p. 239.
[26] Centralbl. f. Bakteriol., II, 1909, 23, p. 672
[27] N. D. Agric. Exper. Station Bull. 116, 1916, p. 348.
[28] Am. Rev. Tuberc., 1919, 3, p. 86.
[29] Jour. Infect. Dis., 1921, 29, p. 233.
[30] Jour. Med. Res., 1905, 14, p. 193.
[31] Jour. Bacteriol., 1921, 6, pp. 447 and 521.
[32] Jour. Path. & Bacteriol., 1916, 20, p. 327.
[33] Jour. Bacteriol., 1919, 4, p. 149.
[34] Jour. Hyg., 1911, 11, p. 341.
[35] Jour. Biol. Chem., 1915, 20, p. 445; Jour. Bacteriol., 1916, 1, p. 15.
[36] Arch. de med. exper. et d'anat. path., 1913, 25, p. 449.

and Onslow.[37] The routine use in this laboratory of such amino acid-rich digests dates from the studies of Stickel and Meyer,[38] whose adequate historical review and description of preparation may be consulted. The anaerobic growth-promoting properties of a combination of beef heart infusion and peptic digest have been found by various workers in this laboratory to aid in isolation from field specimens,[39] and the recovery of organisms attenuated by heat [40] and by antiseptics.[41] Such a medium was, therefore, chosen for this experiment.

Preparation of Medium.—Beef heart medium was prepared as will be described in a subsequent paper. Repeated grindings reduced the size of the muscle particles to a pasty mass, which were returned to their liquor. Approximately 1800 c c. of a mixture of one part of the beef heart medium and two parts of peptic digest [38] were placed in 2-liter Erlenmeyer flasks and sterilized in the autoclave at 115 lbs. pressure for 1 hour on 2 successive days, with incubation at 37 C. for 8 hours prior to the second heating period.

After agitation the meat particles in this medium tended to remain suspended, making possible the removal of representative samples, and the maintenance of a culture medium of almost uniform nitrogen content. The methods of incubation and sampling described by Wolf and Harris [42] were followed. Wolf [43] has clearly stated the difficulties he encountered in the use of these methods with a medium containing meat particles, as follows:

> "During the earlier stages of sampling the solid cooked meat is retained in the fermentation flasks, and consequently the medium becomes more concentrated with respect to protein. As proteolysis proceeds, this protein is digested and the nitrogen is rendered soluble, and can be withdrawn through the sampling tube. Hence it will be found that the latter samples contain concentrations of nitrogen in the form of ammonia and amino acids, which are higher in percentage than the original medium. Consequently it is impossible to calculate the true percentage of nitrogen transformed into ammonia and amino acids."

The effectiveness of the repeated grindings and the thorough agitation before sampling as a means of reducing this source of error to a minimum is perhaps best illustrated by a comparison of the initial total nitrogen with the determinations of total nitrogen on the 10th day of incubation of the B. botulinus, B. sporogenes and B. tetani cultures, as noted in table 1. Calculations show the increase in total nitrogen to be 11%, 16%, and 18%, respectively. Since Wolf [43] made only initial total nitrogen determinations, it is not possible to make a direct comparison. However, a comparison of the sums of the amino acid and ammonia nitrogren found by him with his initial total nitrogen determinations is illuminating. For example, in experiments with B.

[37] Lancet, 1916, 1, p. 9.
[38] Jour. Infect. Dis., 1918, 23, p. 68.
[39] Ibid., 1922, 31, p. 501.
[40] Ibid., p. 650.
[41] Unpublished.
[42] Jour. Path. & Bacteriol., 1917, 21, p. 386.
[43] Ibid., 1918, 22, p. 270.

sporogenes on a cooked meat medium, from which the number of samples removed was comparable to the number removed in the B. sporogenes reported herein, the sums of the amino acid and the ammonia nitrogen only are 72% and 191% larger on the 17th day of incubation than the initial total nitrogen content. On this same basis the figures reported herein for the culture of B. sporogenes show the sum of the amino acid and ammonia nitrogen on the 18th day to be approximately 20% less than the initial total nitrogen content.

A second and it is believed more fundamental source of error which is engendered by the increasing concentration of nitrogen in the mediums as a result of the sampling will be discussed in subsequent paragraphs.

History of Organisms Used.—B. botulinus—Strain 97—Type A.

Isolated in this laboratory September, 1921, from home canned pears received from Yelm, Washington.

B. sporogenes—Strain 46.

Obtained from Professor Hall and described by him.[6] Highly resistant to heat—36 minutes at 105 C.

B. tetani—Strain VTS. Described by Heller.[44]

B. botulinus—Type C.[1] Isolated by Bengtson [45] from the larvae of the green bottle fly, Lucilia sericata. Produces toxin, capable of causing typical symptoms of botulism, but which is not neurtalized by either Type A or B antitoxin. Bengtson found neither liquefaction of gelatin nor coagulation or digestion of casein in 14 days.

Inoculums.—Spores were grown in double strength veal infusion—peptic digest—gelatin for 10 days, as recommended by Esty and Meyer.[46] Twenty c c. amounts of such suspensions, heated for one hour at 80 C., were used for inoculation in each case.

TWO POSSIBLE SOURCES OF ERROR

Size of Inoculums.—It is unfortunate that the B. sporogenes suspension contained more viable organisms per c c. than either the B. botulinus or B. tetani seed cultures. The early reproductive activity of the former, as well as the early chemical activity, is not believed to be entirely attributable to the number of organisms introduced into the experimental flasks. Wolf [43] reported rapid biologic reactions with B. sporogenes inoculums. Previous work with B. botulinus cultures has shown that after the first few hours of rapid multiplication the size of the inoculum exerts little effect. Graham-Smith [47] reports similar results with a staphylococcus culture.

Increase in Nitrogen Concentration Due to Sampling.—Exact chemical data support former observations on the high biologic value

[44] Jour. Infect. Dis., 1922, 30, p. 33.
[45] Pub. Health Rep., 1922, 37, p. 164.
[46] Jour. Infect. Dis., 1922, 31, p. 650.
[47] Jour. Hyg., 1920, 19, p. 133.

of beef heart-peptic digest medium for the 3 anaerobes concerned. The most conclusive evidence is, of course, the high level of growth maintained throughout the first 6 days of the experiment, as shown by plate counts. A comparison of these growth curves (fig. 2) with those for B. botulinus on a similar medium, to be published by one of us (C. C. D.), will show a close correspondence.

The point of difference worthy of mention is the slightly higher growth level maintained in this series of cultures. The 11%, 16%, and 18% increases of total nitrogen in the B. botulinus, B. sporogenes and B. tetani cultures, respectively, due to the retention of meat particles

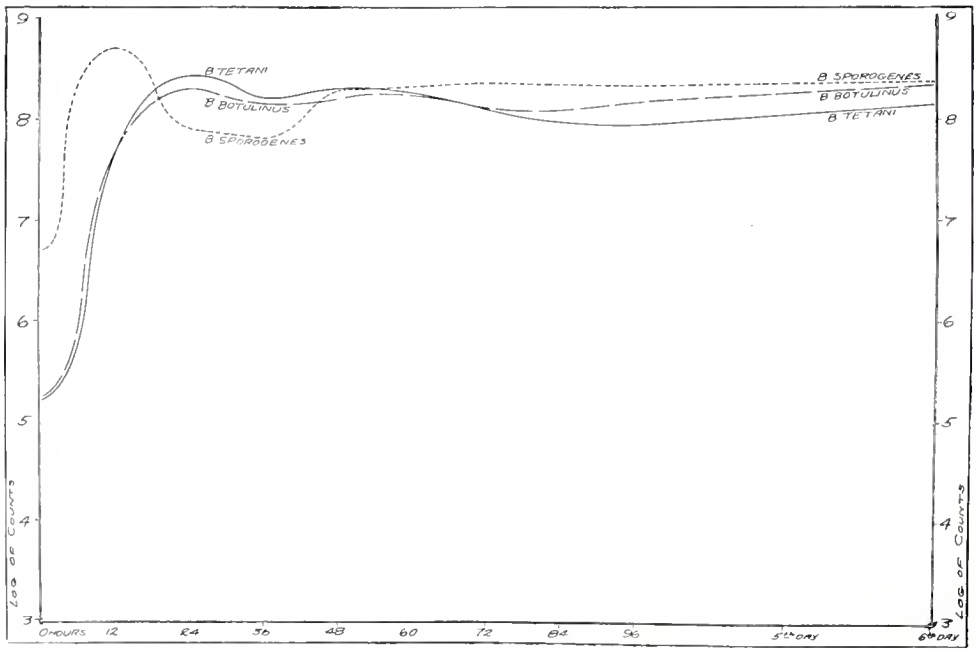

Fig. 2.—Growth of B. botulinus, B. tetani and B. sporogenes in peptic digest-beef heart mince.

during sampling operations have been discussed in relation to the error involved in estimating the percentage of protein hydrolyzed to amino acids and ammonia. Of even more importance, and a relationship which Wolf [43] apparently failed to recognize, is the growth impetus which the increase in nitrogren content of the medium undoubtedly exerted. It may be recalled that Graham-Smith [47] was able to maintain growth of staphylococci at the highest level for two weeks by daily additions of food substances to meat extract cultures. It seems very likely that the greater concentration of protein which follows sampling, and which

subsequently hydrolyzes, may act in the same way, and the results obtained with the small increases of total nitrogen (less than an average of 2% increase after each sampling) confirm the hypothesis. In a substratum of lower biologic value such a small percentage increase would probably have little effect, as was the case in the medium used by Graham-Smith. The increases of more than 72% and 191% in the nitrogen content, reported by Wolf, undoubtedly acted as powerful growth stimulants.

Experimental

Methods of incubation and sampling were identical with those to be reported in a later paper. Samples for chemical determinations and plate counts were removed from the large culture flasks at 12-hour intervals for the first 3 days of incubation and then less frequently. Unless otherwise stated, methods of chemical analysis to be described later were adhered to. Samples removed from the flasks immediately after planting served as controls. Determinations on the morning samples were made promptly after removal; those on the night samples, with the exception of plate counts, which were made immediately, on the following morning. During the interim the material was kept in the icebox.

Total Nitrogen.—Total nitrogen determinations were made on the controls and the 10-day cultures. Calibrated pipets were invariably used to measure the 2 c c. specimens used for analyses. Duplicate determinations on the control samples from the B. botulinus culture checked exactly; those for B. sporogenes and B. tetani failed to give quite such satisfactory results. There were variations of 0.8 c c. of N/10 alkali for B. sporogenes and 0.6 c c. for B. tetani in duplicate determinations. When each set was averaged, however, the cultures had the same total nitrogen content as the B. botulinus culture. Duplicate determinations on the 10-day culture of B. botulinus checked; for B. sporogenes there was a variation of 0.4 c c., and for B. tetani, 0.2 c c. Averages of the two sets of determinations are reported.

The total nitrogen for the B. botulinus, B. sporogenes and B. tetani cultures had increased 11%, 16% and 18%, respectively, on the 10th day. In view of the fact that a total of 11 samples had been removed from each culture, these increases are relatively small. These figures will be referred to again in subsequent paragraphs.

Nonprotein Nitrogen.—Folin and Wu's [48] tungstic acid precipitation method was followed. After such precipitation bacterial culture filtrates may contain peptids, amino acids, ammonia, creatinin, amines and probably small amounts of uric acid, phenols, purin bases and various other compounds. While the exact size of the molecules precipitated is not known, total nitrogen determinations made on duplicate 20 c c. portions of the protein-free filtrates indicate that fairly definite nitrogen compounds must be removed.

The nonprotein nitrogen fractions of the 3 cultures increased so that by the 6th day 96% of the nitrogen of the original culture medium was resident in compounds of soluble form. Kendall [19] identified 94% of the nitrogen of 4-day old B. proteus cultures as nonprotein nitrogen, and on the 14th day the amount had decreased to 83%, which suggests that some of the nitrogen had been

[48] Jour. Biol. Chem., 1919, 38, p. 81.

TABLE 1

GROWTH OF B. BOTULINUS TYPES A AND C, B. SPOROGENES AND B. TETANI ON PEPTIC DIGEST-BEEF HEART MINCE

	Time	Total Nitrogen, Mg.	Nonprotein N., Mg.	Amino N., Mg.	Ammonia N., Mg.	Volatile Acid N/10, C c.	Titrable Acid N/1, C c.	P_H "C"	Gas per Liter, C c.	Creatinin, Mg.	Counts per C c.
B. botulinus Strain 97 Type A	0 hours	970	427	174	44	4	3.1	6.6	...	45.0	172,000
	12 hours	...	532	194	69	14	3.3	6.6	92	....	50,000,000
	24 hours	...	686	272	237	81	...	6.8	467	37.0	199,300,000
	36 hours	...	...	243	269	105	3.9	6.9	740	....	142,500,000
	48 hours	...	...	280	302	...	4.3	6.8	1000	....	160,000,000
	60 hours	...	...	292	312	138	4.5	6.9	1075	....	175,000,000
	72 hours	...	...	325	342	142	5.0	6.8	1200	....	132,000,000
	96 hours	...	868	341	353	128	4.6	...	....	35.0	146,000,000
	120 hours	...	...	...	...	130	...	6.8	....	....	.....
	6 days	...	945	327	373	...	...	6.9	....	39.0	207,000,000
	10 days	1078	...	354	394	171	4.9	...	....	37.2	.....
	18 days	...	...	366	420	...	...	7.2	....	....	.....
B. sporogenes Strain 46	0 hours	970	427	174	44	...	3.2	6.6	....	45.0	5,000,000
	12 hours	...	763	240	167	54	3.6	7.0	205	33.5	500,000,000
	24 hours	...	798	272	249	92	...	6.9	544	33.4	62,000,000
	36 hours	...	...	272	286	111	3.7	7.0	736	....	64,000,000
	48 hours	...	...	261	322	...	4.4	7.1	930	....	189,000,000
	60 hours	...	...	201	...	124	4.2	...	970	40.2	234,000,000
	72 hours	...	...	274	361	130	5.0	7.0	1020	....	230,000,000
	96 hours	...	861	282	368	135	6.0	6.8	....	....	212,000,000
	120 hours	...	...	...	...	...	...	...	....	....	.....
	6 days	...	931	315	397	152	...	6.9	....	42.0	236,000,000
	10 days	1127	...	317	417	158	5.0	7.0	....	38.0	.....
	18 days	...	...	343	429	...	...	7.0	....	....	.....
B. tetani Strain V. T. S.	0 hours	970	427	174	44	4	3.2	6.6	....	37.0	100,500
	12 hours	...	455	174	64	21	3.1	6.6	121	31.5	50,000,000
	24 hours	...	657	186	121	49	...	6.8	383	13.8	268,000,000
	36 hours	...	...	203	145	57	3.3	6.8	532	....	164,000,000
	48 hours	...	...	232	176	...	4.1	6.9	685	....	63,000,000
	60 hours	...	...	201	280	120	4.9	6.9	750	10.0	205,000,000
	72 hours	...	...	184	390	174	...	6.9	780	....	134,000,000
	96 hours	...	875	206	435	...	5.4	7.0	....	9.4	87,000,000
	120 hours	...	...	...	...	...	...	...	....	....	.....
	6 days	...	945	258	472	...	...	7.0	....	7.5	139,000,000
	10 days	1148	...	275	510	246	6.0	7.2	....	6.7	.....
	18 days	...	...	277	530	...	...	7.6	....	....	.....
B. botulinus Type C	0 hours	970	427	...	44	...	...	6.6	....	....	.....
	24 hours	...	511	233	39	14	...	...	....	44.0	.....
	72 hours	...	...	227	53	23	...	6.0	....	45.0	.....
	6 days	...	...	261	53	28	4.7	5.8	....	42.7	.....
	30 days	...	...	292	61	...	4.6	5.6	....	....	.....

synthesized into bacterial protein. No such inference can be drawn from our results. The significance of nonprotein nitrogen will be discussed in later paragraphs.

Ammonia Nitrogen.—An almost alarming number of methods for ammonia determinations exists. Allan and Davidson,[49] after comparing colorimetric and titrametric methods, write: "Titration procedures have added advantages over colorimetric in that they are applicable to a wide range of nitrogen changes and hence by a regulation of the size of the sample the error due to aliquoting may be kept smaller." The aeration method of Folin[50] has been used generally for bacterial metabolism studies. The technic, however, has been modified

[49] Ibid., 40, p. 182.

[50] Ztschr. f. Physiol. Chem., 1902, 37, p. 161.

by different workers. Sears[24] used $CaOH_2$ for the liberation of ammonia; Kendall,[51] a saturated solution of Na_2CO_3 and potassium oxalate. Waksman[20] found after aeration for 3 hours with Na_2CO_3 as much as 25% additional ammonia nitrogen could be removed by aerating further with 1 gm. of NaOH and 10 gm. of NaCl. Wolf and Harris[14] used Shaffer's vacuum distillation method.[52]

In previous experiments, seemingly inconsistent results for ammonia determinations have been encountered occasionally when Na_2CO_3 was used as a reagent. Since there was involved not only the question of the complete recovery of the ammonia from the cultures, but also the question of the decomposition of the constituents of the medium by the reagents, it was believed highly important to make a comparative study of the methods most commonly employed for ammonia determinations. While further investigation of this problem is in progress, the results of some of the determinations are suggestive and are given in table 2. Na_2CO_3 and NaOH plus NaCl are equally efficient in removing the ammonia from ammonium chloride and from triethylamine. From 4% peptone solutions higher results were obtained with the latter. The same greater efficiency of the NaOH plus NaCl is evident with solutions of ammonium chloride plus peptone. When phosphate is added lower results with both reagents may be observed. Steel[53] found ammonium-magnesium-phosphate which may exist in peptone solutions, was not decomposed by Na_2CO_3. He also found that common urinary nitrogen compounds, such as urea and uric acid, were not decomposed by NaOH plus NaCl; nor did the amino acids he aerated with NaOH yield ammonia.

Because there are no generally accepted standards for time of aeration and strength of alkali necessary for the complete removal of ammonia from culture mediums preliminary tests were undertaken. Five c c. of concentrated NaOH or 1 gm. of Na_2CO_3 proved as efficient as larger amounts in liberating the ammonia from 5 c c. of cultures. Results for one-half, 1, 2 and 3 hour aerations show that 2 hours is a sufficient aeration time.

Parallel ammonia determinations were made by the aeration method, using Na_2CO_3 and NaOH plus NaCl, and the Shaffer vacuum distillation method. Determinations by the latter method were time-consuming and the results less reliable than by either form of the aeration method. Calculations show that NaOH plus NaCl liberated, on an average, 13.5% more ammonia than did Na_2CO_3. The source of this additional ammonia is not known. However, on account of its greater efficiency NaOH plus NaCl has been used.

For routine ammonia determinations 5 c c. of culture was measured into an 8 × 1 inch Folin urea tube. To this 15 c c. of concentrated NaCl solution, a few drops of caprylic (Kahlbaum) alcohol and 5 c c. of concentrated NaOH were added. The ammonia was driven by aeration into a measured amount of N/10 H_2SO_4. The use of N/10 solutions was necessary because of the anticipated large changes in ammonia content between determinations.

Amino Acids.—None of the methods available accounts for all the nitrogen of the amino acids. By the Van Slyke method[54] the nitrogen of the heterocyclic amino acids and arginine is incompletely removed, and that of the epsilon group of lysine reacts slowly. By the recently developed Folin colorimetric method[55] the nitrogen of an equal number of amino acids fails to react quantita-

[51] Jour. Infect. Dis., 1922, 30, p. 211.
[52] Am. Jour. Physiol., 1908, 18, p. 330.
[53] Jour. Biol. Chem., 1910, 8, p. 365.
[54] Ibid., 1913, 16, p. 121.
[55] Ibid., 1922, 51, p. 377.

tively. Only after determination of the specific amino acids in mediums before and after growth will strictly quantitative results concerning the amino acid metabolism of bacteria be attained; especially is this true since there is considerable evidence of selective use of amino acids by bacteria.

The Folin method used by De Bord [21] for the determination of amino acids in 2% peptone cultures has, it is true, certain advantages over the Van Slyke method; the question of optimum time for complete deaminization is obviated. In dealing, however, with material in which the amino acid nitrogen is apt to

TABLE 2

COMPARISON OF AMMONIA DETERMINATIONS

Substance Tested (Volume 25 C c.)	1 Gm. Na_2CO_3, N/50 Acid Neutralized, C c.	5 C c. Concentrated NaOH + 10 Gm. NaCl, N/50 Acid Neutralized, C c.	Shaffer Method,* N/50 Acid Neutralized, C c.
Control	0.25	0.37	....
NH_4Cl	16.2 16.2	16.4 16.4	....
4% peptone (15 c c.)	2.0 2.0	3.7 3.9	....
NH_4Cl + peptone (15 c c.)	18.2 18.4	20.3 20.4	....
NH_4Cl + 10% phosphate + peptone (15 c c.)	13.2 13.3	18.4 18.4	....
Amine (triethyl)	34.1 34.1	34.1 34.2	....
Control veal infusion peptone (5 c c.)	6.0 6.1	7.0 7.1	....
40 hour culture veal infusion peptone (5 c c.)	10.3 10.3	11.5 11.3	....
46 hour culture veal infusion peptone (5 c c.)	13.6 13.4	16.2 16.1	22.5 20.5
52 hour culture veal infusion peptone (5 c c.)	17.6 17.6	21.2 21.0	33.0 39.0
67 hour culture veal infusion peptone (5 c c.)	21.4 21.4	24.4 24.5	....
96 hour culture veal infusion peptone (5 c c.)	24.5 24.5	28.0 28.0	....

* 10 c c. used for determinations.

change as much as 50 mg. per 100 c c. between determinations, the errors involved in dilution technic practically prohibit this method replacing that of the Van Slyke for amino acid determinations.

Van Slyke [64] considered 3 minutes at 20 C. ample time for complete deaminization of the alpha amino group of the amino acids. Since the laboratory temperature averaged not far from 20 C. the 3-minute interval was arbitrarily chosen. Occasionally in amino acid-rich material such as is used in this experiment, as many as 4 determinations were made before satisfactory checks were secured. In such cases averages have been reported. This method involves, it is believed, no appreciable error, even if the variations are due to differences in

degree of deaminization, since the determinations were invariably made under the same conditions. The large number of determinations made by the Van Slyke method in this laboratory indicates that the 3-minute interval yields consistent results. It should be emphasized, however, that greater significance should be placed on the comparative rather than on the absolute value of amino acid content in bacterial metabolism studies.

Creatinin.—Creatinin determinations were made on the tungstic acid filtrates of the cultures by Folin's method,[56] using a pure creatinin standard. The results show changes in the B. botulinus and B. sporogenes cultures, which are hardly large enough to be significant and which may be due to experimental error. The creatinin gradually disappeared from the B. tetani culture, indicating that this organism, in contrast to B. botulinus and B. sporogenes, is able to destroy this compound. Sears [24] found creatinin destroyed by numerous aerobes. It is of interest to note, however, that Mellanby [57] found that B. tetani was the only anaerobe he studied which completely destroyed creatin, the hydride of creatinin.

Gas Production.—On the 12th hour, B. sporogenes had formed twice as much gas as either B. botulinus or B. tetani. The gas production of B. botulinus and B. sporogenes for the first 60 hours, when charted, gives remarkably similar curves. After that time, while gas was produced, the heavy sampling had increased the gas space so that readings were not considered accurate enough to record.

Acid Production.—The production of H_2S in this type of medium interferes with the electrometric determination of the hydrogen-ion concentration. The reaction changes therefore were followed colorimetrically. The brown coloring matter in the mediums made color determinations somewhat difficult, and no significance is placed on changes of less than 0.2 P_H. The large amount of buffer material developed as the proteolytic activity of the organisms proceeded, permitted only slight changes in reactions.

Direct Titrations.—Such titrations of cultures of this kind gave an absolutely misleading impression of the acid production. In combination with other findings, determinations of titratable acid have some value.

Ten c c. of the culture plus 1 c c. of phenolphthalein plus 90 c c. of boiled distilled water were titrated with N/10 NaOH to match P_H 8.6 in the phenol red color scale. The results are given as c c. of N/1 acid per 100 c c. of medium.

The titratable acidity of the B. botulinus and B. sporogenes cultures progressively increased to +5 and +6, respectively, after which the amounts in both cultures decreased. There was a slow increase in titratable acidity in the B. tetani culture throughout the experimental period. Bunker [58] found the addition of Witte's peptone to veal infusion increased the titratable acidity in direct proportion to the amount of peptone added. The increase in titratable acidity in sugar-free mediums is probably a measure of the COOH groups of proteins or their cleavage products. The changes in titratable acidity in the B. sporogenes cultures are in agreement with those found by Kendall [17] in nutrient broth and in gelatin cultures.

Volatile Acids.—The methods for the removal and identification of volatile acids will be given in detail in a future article. The accumulation of volatile acids in the cultures of the 3 organisms followed in a general way the ammonia

[56] Ibid., 1919, 38, p. 81.
[57] Jour. Physiol., 1908, 36 p. 447.
[58] Jour. Bacteriol., 1919, 4, p. 379.

TABLE 3

VOLATILE ACID PRODUCED IN PEPTIC DIGEST-BEEF HEART MINCE

	60 Hour Cultures						18 Day Cultures					
	B. botulinus		B. sporogenes		B. tetani		B. botulinus		B. sporogenes		B. tetani	
	1st Fraction	2d Fraction	1st Fraction	2d Fraction	1st Fraction	2d Fraction	1st Fraction	2d Fraction	1st Fraction	2d Fraction	1st Fraction	2d Fraction
C c. N/10 barium hydroxide to neutralize volatile acids	28.9	16.4	33.5	15.4	30.4	16.1	69.8	28.7	45.6	23.4	47.2	26.4
Percentage of volatile acids in fractions of distillate (Duclaux method) — 10 c c.	22.5	12.2	22.7	11.5	19.1	11.8	24.1	16.7	24.2	12.8	19.5	12.5
20 c c.	40.0	23.2	41.5	23.4	33.6	23.0	43.2	29.3	42.1	23.1	35.6	23.1
30 c c.	53.0	33.5	56.1	33.1	46.0	33.5	57.1	40.0	56.6	33.8	49.0	33.7
40 c c.	64.0	42.6	67.2	42.2	56.3	42.8	69.0	50.1	67.2	42.8	60.0	43.5
50 c c.	72.4	51.2	75.5	51.4	65.8	51.5	78.0	59.0	75.0	51.7	70.0	53.0
60 c c.	79.6	61.0	82.0	61.0	74.0	60.8	84.8	67.6	81.0	60.2	77.5	61.8
70 c c.	85.0	69.5	88.0	69.5	81.4	70.0	90.0	75.8	86.6	69.4	84.4	70.8
80 c c.	90.5	79.4	92.5	80.0	87.6	77.0	94.0	83.2	91.0	78.2	91.8	79.5
90 c c.	95.0	88.0	96.4	89.0	93.0	88.2	97.5	91.1	95.4	88.0	97.2	88.6
100 c c.	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Ratio of acids present from titration sequence	2 valeric 1 acetic	1 butyric 2 acetic	2 valeric 1 acetic	1 butyric 2 acetic	1 valeric 1 acetic	1 butyric 2 acetic	3 valeric 1 acetic	2 butyric 1 acetic	4 valeric 1 acetic	1 butyric 2 acetic	1 valeric 1 acetic	1 butyric 2 acetic
	Gm.		Gm.	Gm.	Gm.	Gm.	Gm.		Gm.	Gm.	Gm.	Gm.
Volatile acids as barium salts	0.4693	(Lost)	0.5892	0.2465	0.4995	0.2536	1.0822	(Lost)	0.7558	0.369	0.6546	0.3391
$BaSO_4$ equivalent found	0.3643	.....	0.4477	0.3075	0.3948	0.2176	0.7967	.....	0.5383	0.3315	0.5336	(Lost)
Calculated as Ba acetate	0.399	.....	0.490	0.336	0.432	0.2378	0.872	.....	0.580	0.363	0.584	.....
Calculated as Ba propionate	0.445	.....	0.524	0.373	0.479	0.264	0.967	.....	0.646	0.425	0.650	.....
Calculated as Ba butyrate	0.487	.....	0.596	0.410	0.505	0.287	1.06	.....	0.710	0.443	.....	.....
Calculated as Ba valerate	0.533	.....	0.650	0.612	0.575	.....	1.16	.....	0.775	.....	0.776	.....
Calculated from ratio of acids found by titration	0.487	.....	0.597	0.2445	0.503	0.2565	1.088	.....	0.736	0.387	0.680	.....
Difference between volatile acids barium salts, found and calculated	0.008	.....	0.007	0.002	0.0035	0.0029	0.0058	.....	0.0198	0.018	0.026	.....

production (table 1). This was especially conspicuous in the case of the B. tetani culture where both ammonia and volatile acid accumulations, compared with those of the other organisms, were much delayed. On the 10th day the proportion of volatile acid to ammonia was greater in the B. tetani culture than in either of the others. The fact that B. tetani formed less gas may account for this, for where equal protein degradation has occurred, if less carbon is given off as CO_2 more is probably retained in the acid form.

The acids in the 60-hour and 18-day cultures were identified in accordance with the data presented in detail in table 3. As may be noted therefrom, the identification procedure varied somewhat from that usually employed. The barium salts of the volatile acids secured by titration were dried, weighed, and converted to barium sulphate and again weighed. The entire barium content was then computed as acetate, propionate, butyrate and valerate. A combination of these salts in the molecular proportions indicated by the titration figures approximated closely the weights actually found, and offered further proof of the correctness of the interpretation of the acid contents of the culture mediums by means of the fractional distillation results.

TABLE 4

NONVOLATILE ACID PRODUCED IN PEPTIC DIGEST-BEEF HEART MINCE

	N/10 Nonvolatile Acid per 100 C c. of Culture, C c.	Nonvolatile Acids of Aliquot Part as Barium Salt, Gm	Barium Sulphate Equivalent		N/10 Lactic Acid per 100 C c. Culture Calculated from $BaSO_4$, C c.
			Found, Gm.	Calculated for Lactic Acid, Gm.	
Control medium	6.2	.....	.....	.....	....
B. botulinus 96 hour culture.	53.6	0.1243	0.072	0.0556	49.4
B. botulinus 10 day culture..	56.8	0.2590	0.1790	0.141	62.4
B. sporogenes 10 day culture.	60.0	0.2623	0.608	0.126	56.0
B. tetani 96 hour culture.....	72.0	0.1595	0.1032	0.0798	70.8

The acids in the 60-hour cultures of B. botulinus and B. sporogenes were almost similar, while in the B. tetani culture more acetic acid was undoubtedly present. The acids in the 18-day cultures of B. botulinus and B. sporogenes differed from those in the young cultures in that there was a greater proportion of acids of higher molecular weight. No change in the proportion of the acids in the 60-hour and the 18-day culture of B. tetani could be detected.

While a too specific interpretation of results should be avoided, a few figures are cited to show the contrast between the acids in the 18-day cultures of B. botulinus and B. tetani. A representative sample from the B. botulinus culture contained 50.7 c c. N/10 valeric and 28.4 N/10 acetic, with some acid of intermediate molecular weight, while that from the B. tetani culture contained 23.6 c c. N/10 valeric and 41.2 c c. N/10 acetic, with a smaller amount of intermediary acid. Similar differences have been observed before. In one meat culture of B. tetani the acid was identified as almost pure acetic. Heller [11] reported a lack of butyric acid odor in B. tetani cultures.

Nonvolatile Acids.—While only a few determinations were made for fixed acids, the results give an idea of the ratio of fixed to volatile acids. Data in table 4 show that less than one third of the acids in the cultures was nonvolatile, and the ratios between the two types of acids were similar in the three cultures. For identification of the fixed acid the titrated acid salt was evaporated, weighed, and in turn converted to barium sulphate. From the weight of the barium

portion of the barium sulphate, the theoretical amount of barium lactate possible was calculated. These figures do not agree closely enough with the amount of barium salt actually recovered to justify the assumption that the acid present was all lactic, and further work to establish the identity of the fixed acid is necessary. The results indicate, however, that only small amounts of acids of higher molecular weight were present.

PROTEOLYSIS AS MEASURED BY AMMONIA AND AMINO ACID NITROGEN OF THE CULTURES

Ammonia Nitrogen.—A comparison of the mg. of ammonia nitrogen in 100 c c. amounts of the 48-hour cultures of B. botulinus, B. sporogenes and B. tetani (table 1) shows a ratio of 302: 322: 176, respectively. Using this as a measure of proteolytic activity, B. sporogenes and B. botulinus may be considered possessed of approximately equal proteolytic power, while B. tetani is only weakly so provided. If the comparison be made at the end of 72 hours' incubation the ratio is 342: 361: 390, respectively, while at the end of 18 days the figures have changed to 420: 429: 530, showing the greatest proteolytic power to reside in B. tetani.

The ammonia content of each of the cultures has increased at a rather rapid rate, and there is no indication of any synthesis by which ammonia is being removed. During the first 6 days at least, the numbers of living organisms have remained consistently high in all of the cultures, which fact, together with the vigorous ammonia production during all but the first few hours of incubation, is evidence of a prolonged energy phase.

Amino Acid Nitrogen.—Forty-eight hour amino acid nitrogen determinations are in the ratio of 280: 261: 232, for B. botulinus, B. sporogenes and B. tetani, respectively. If amino acid be a measure of proteolytic activity, the conclusion is justified, as was the case with 48-hour ammonia determinations, that B. botulinus and B. sporogenes are more strongly proteolytic than B. tetani. Determinations on the 72nd hour show a ratio of 325: 274: 184, by which it may be concluded that B. botulinus is strongly proteolytic, B. sporogenes moderately proteolytic and B. tetani weakly proteolytic. Again, on the 18th day of incubation the ratio is 366: 343: 277, leaving the conclusions as before.

A study of the two sets of ratios is instructive. They have been graphically represented in fig. 3. If ammonia nitrogen is used as a measure of proteolysis, B. tetani changes after 48 hours' incubation from a weakly proteolytic organism to the most proteolytic of the 3,

while if amino acid nitrogen is used as a measure, B. tetani remains weakly proteolytic in comparison with B. botulinus and B. sporogenes. This "balance" between ammonia nitrogen and amino acid nitrogen is shown too by the figures for B. botulinus and B. sporogenes. On the 18th day the ammonia content of the B. botulinus culture is slightly less than that of B. sporogenes, while the amino acid contents of the two cultures are just the reverse, although the differences are not significant.

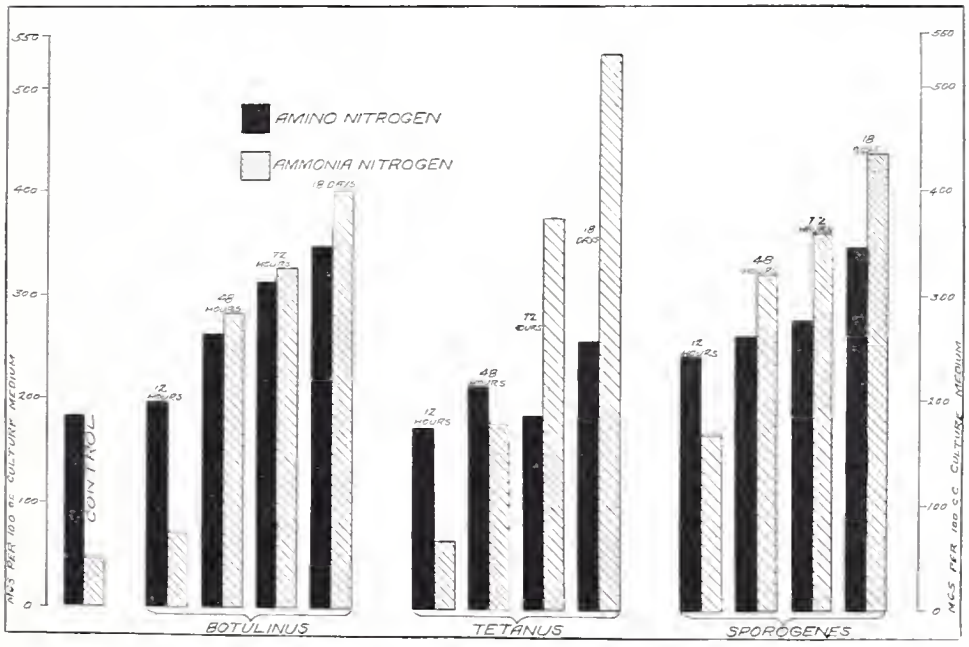

Fig. 3.—The production of amino-acid and ammonia by B. botulinus, B. tetani and B. sporogenes on peptic digest-beefheart mice.

The late vigorous ammonia production by the tetanus culture was surprising. If the hourly rate is charted, it will be found that the period of most active production is between the 48th and 72nd hour of incubation. This period followed one of less vigor, during which time the crest of the ammonia curve was less than one half the height of that of the second wave. It hardly need be restated that the question of contamination has been eliminated, although there has been kept in mind Kendall, Day and Walker's [17] conclusion, "From the standpoint of the metabolism of anaerobic bacilli, it may be stated dogmatically that any culture containing living bacteria which shows little proteolysis for a week or 10 days followed by a sudden and note-

worthy jump in the chemical evidences of proteolysis is contaminated with B. sporogenes, or somewhat less commonly, another of the proteophilic anaerobic bacilli."

CLOSER ANALYSIS OF METABOLIC ACTIVITIES

To facilitate a comparison of the various nitrogen products produced by the organisms, table 5 is included. An attempt is made to measure the actual proteolytic activities of the organisms by deducting the amounts of breakdown products originally present from the amounts found after varying periods of incubation. It should be emphasized, therefore, that the figures in table 5 represent not the total amounts of different products present in the medium at any one time, but only a measure of those which have been formed by the organisms.

TABLE 5

PROTEIN DEGRADATION PRODUCTS IN CULTURES OF B. BOTULINUS, B. SPOROGENES AND B. TETANI

Hour of Incubation	Ammonia Nitrogen			Amino Nitrogen			Sum of Amino Acids and Ammonia Nitrogen			Nonprotein Nitrogen			Higher Protein* Degradation Products		
	B, Mg.	S, Mg.	T, Mg.	B, Mg.	S, Mg.	T, Mg.	B, Mg.	S, Mg.	T, Mg.	B, Mg.	S Mg.	T, Mg.	B, Mg.	S Mg	T, Mg.
0	44	44	44	174	174	174	218	218	218	427	427	427	209	209	209
12	25	123	21	20	66	0	45	189	21	105	336	28	60	147	7
24	193	205	77	98	98	12	291	303	89	259	371	230	—32	68	141
96	309	324	391	167	108	32	476	432	423	441	434	444	—35	2	21
6 days	329	353	428	153	141	84	482	494	512	518	504	518	36	10	6

B = B. botulinus, S = B. sporogenes, T = B. tetani.
* This fraction consists of all soluble nitrogenous material except amino acids and ammonia.

In spite of the vigorous growth, by the 24th hour in each case, as shown by plate counts, B. tetani has produced over and above its consumption, practically no amino acids. The ammonia, however, has increased moderately, and this may be interpreted, according to Kendall, as an indication of considerable intracellular activity, but which after all, is only one third that of B. sporogenes and B. botulinus. Since only 89 mg. of the 230 mg. of the nonprotein nitrogen produced by the tetanus culture by the 24th hour have been identified as amino acid and ammonia, approximately 141 mg. may be assumed to be in the form of higher nitrogen degradation products. The activity of the tetanus culture during the first 24 hours therefore has been largely directed toward attacking the protein of the medium, and neither ammonia nor amino acid production can be an adequate measure of its

proteolytic activity. On the contrary, B. botulinus has not attacked native proteins as rapidly as it has utilized the higher degradation products, since it has used 32 mg. of nitrogen from its original store of these materials. B. sporogenes also utilized large quantities of higher protein degradation products during the period ending on the 24th hour, but owing to accumulation of such products during the first 12 hours of incubation, in spite of its vigorous ammonia production, it did not need to draw on its original store.

The period from the 24th to the 96th hour represents one of considerable activity in the 3 cultures (energy phase?) during which time B. botulinus attacked protein and the higher degradation products at an equal rate; B. sporogenes utilized considerably more of the higher degradation products than it produced and B. tetani not only utilized the greatest amount of higher degradation products previously accumulated, but attacked native protein more violently than either B. sporogenes or B. botulinus. The pronounced difference in the attacks on native protein is shown by the ratios of nonprotein nitrogen increase, 182 : 63 : 214, respectively, for B. botulinus, B. sporogenes and B. tetani during this period. On the 96th hour the total nonprotein nitrogen content of the 3 cultures is approximately equal, which is evidence of proteolytic powers of equal intensity.

Determinations made on the 6th day of incubation indicate an almost complete destruction of the native proteins in all of the cultures, and there is evidence of practically equal moderate activity in attacks during the preceding 48 hours on the small protein moiety of the mediums. B. tetani, however, has produced 52 mg. of amino acids from the higher complexes, in contrast to a slight loss by B. botulinus and a slight gain by B. sporogenes.

These comparisons again lead inevitably to the conclusion that neither the amount of amino acids nor ammonia is a reliable measure of proteolysis. While a sum of these two gives a much more reliable criterion, as suggested by Sears,[24] the fluctuations in the amounts of higher protein degradation products, the "polypeptid" fraction of Kendall[51] make it imperative that the amount of nonprotein nitrogen be determined if accuracy is desired. Such determinations have shown that B. botulinus, B. sporogenes and B. tetani are equally proteophilic when grown for periods of from 4 to 6 days on peptic digest-beef heart mince. The proteophilic tendency of the 3 organisms is still more striking when it is remembered that the medium used was rich in

amino acids and polypeptids, and that at no time was it probable that the breakdown of protein was necessary to provide for building stones and energy needs.

Changes in Reaction of the Cultures.—During the first 10 days of incubation, B. botulinus, B. sporogenes and B. tetani cultures exhibited a slight tendency to become progressively more alkaline. It is in 18-day cultures only that B. tetani has produced a differential alkaline reaction of P_H 7.6. Using alkalinity as a measure of proteolysis, B. tetani must be classed as considerably more proteolytic than either B. sporogenes or B. botulinus with reactions of P_H 7.0 and 7.2, respectively. In contrast, the reaction of type C. B. botulinus became progressively more acid, reaching a P_H of 5.8 on the 6th day of growth. This acid reaction is not unexpected in view of the lack of proteolytic activity, as shown by various determinations for protein degradation products.

Gas Production.—The amounts of gas formed by the three organisms, under the conditions of the experiment, are quite different, yet they seem not to bear any direct relationship to nitrogenous metabolic products. This is not in accordance with the conclusion of Wolf and Harris,[14] who found in the absence of carbohydrate that ammonia and gas production in cultures of B. sporogenes were closely related. It is, of course, premature at this time even to hazard a guess as to possible differences in sources or composition of the gases formed. Attention, however, should be called to the larger amounts of volatile acids in the B. tetani culture which also shows the smallest amount of gas. Retention of larger amounts of carbon in volatile acids might be expected to decrease that available for carbon dioxide.

Toxin Tests.—A few toxin tests were made during the course of the experiment, and are of interest particularly because of the proof they afford of the virulence of the B. botulinus and B. tetani strains as well as the strain isolated by Bengtson. On the 96th hour of incubation B. botulinus had produced a toxin potent enough to be lethal for white mice when 1 c c. of a dilution of 1 : 10,000 of the centrifuged culture fluid was injected intraperitoneally. At the same time 1 c c. of a 1 : 100 dilution of the B. tetani culture was lethal when injected intramuscularly. B. botulinus, Type C, grown on identical mediums has repeatedly yielded a toxin of 1 : 10,000 potency. This is of special interest since the biochemical determinations show the organisms decidedly nonproteolytic, indicating a marked exception to the

conclusion of Heller [59] who writes, "The more proteolytic a pathogen is, the more powerful is its toxin." Results reported herein also call attention to the opinion of the British Medical Committee [60] that ". . . the truly proteolytic organisms are not those which produce potent toxins"; and to Kendall, Day and Walker's [20] statement, "So far as available information indicates, true toxin formation appears to be incompatible with marked proteolysis."

SUMMARY AND CONCLUSIONS

Pure cultures of B. botulinus, B. sporogenes and B. tetani were grown from the spore stage on a medium of high biologic value. Plate counts have shown the maintenance of approximately the same high levels of growth by the 3 organisms for the first 6 days of the experimental period.

A similarity in ammonia accumulation was noted in cultures of B. botulinus and B. sporogenes. Ammonia accumulation during the latter part of the experiment was more marked in the B. tetani culture.

Amino acid nitrogen accumulations in the B. botulinus and the B. sporogenes cultures were almost similar in amount, while the dissimilarity of the intermediary metabolism of the B. tetani culture is shown by a smaller accumulation of amino acid nitrogen.

Nonprotein nitrogen determinations on the 6th day showed that practically all the protein of the 3 cultures had been degraded to soluble nitrogen compounds.

Further evidences of the specificity of the chemistry of the nitrogenous metabolism of B. tetani cultures as compared with B. botulinus and B. sporogenes is afforded by creatinin, volatile acid and gas determinations. B. tetani utilized practically all of the creatinin originally in the medium, while B. botulinus and B. sporogenes lacked this ability. B. tetani formed less gas than either B. botulinus or B. sporogenes, while there was a greater accumulation of volatile acids by the former. The acid produced by B. tetani was predominantly acetic, while the greater proportion of that formed by B. botulinus and B. sporogenes was of higher molecular weight.

Practically equal amounts of fixed acids were formed in the three cultures.

[59] Jour. Infect. Dis., 1920, 27, p. 383.
[60] Brit. Med. Res. Com., Special Report, Ser. No. 39, 1919.

B. botulinus, type C, isolated by Bengtson, showed little chemical activity in a medium of high biologic value for B. botulinus, Type A, B. sporogenes and B. tetani.

It is believed that successive nonprotein nitrogen determinations are the best means available for determining bacterial proteolysis. Such determinations have shown B. botulinus, B. sporogenes and B. tetani to be equally proteolytic when grown on peptic digest-beef heart mince.

EFFECT OF GLUCOSE ON BIOCHEMICAL ACTIVITIES, INCLUDING GROWTH AND TOXIN PRODUCTION OF B. BOTULINUS *

STUDIES ON METABOLISM OF ANAEROBIC BACTERIA II

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The "protein-sparing" power of utilizable carbohydrates in bacterial metabolism manifests itself in numerous ways. The utilization of sugar for energy by the cholera vibrio, B. coli and B. proteus, so changes the metabolism that indol is not formed; B. diphtheriae fails to produce a potent toxin; and the addition of glucose to cultures of strongly proteolytic organisms has been found to prevent the formation of proteolytic enzymes. In fact, Kendall [1] has formulated the theory that "Specificity of action of the vast majority of bacteria depends upon their utilization of protein or protein derivatives for energy." In view of the dynamic protein-degrading ability and the exceedingly strong toxin-producing power of B. botulinus, an opportunity was chosen to determine at short, successive intervals the toxin titers and chemical composition of, and number of viable organisms in, mediums of high biologic value identical in every way except one contained utilizable carbohydrate. Moreover, it was anticipated that some light might be thrown on some of the more intricate phases of bacterial metabolism not marked by such specific metabolic products as toxin, indol, or proteolytic enzymes.

Rubner [2] established the similarity of the growth and metabolic processes of bacteria with those of animals. By means of direct calorimetry he determined the caloric value of culture mediums before and after the growth of organisms. These determinations he checked by direct measurements of the heat generated during various periods of incubation. He also determined the nitrogen and sulphur contents of the bacterial mass removed from the culture fluids, and so had a measure of growth or synthesis. He found approximately four times as much energy was used for "Umsatz" as for "Ansatz." That is, during

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[1] Am. Jour. Med. Sc., 1918, 156, p. 157.
[2] Archiv. f. Hyg., 1906, 57, pp. 161 and 244.

the early hours of incubation the cultures were marked by the reproductive character of the cells, which was followed by a long period of active existence in which the cells continued to function metabolically, but not to reproduce. He concluded the optimum temperature for reproduction was not necessarily the same as for fermentation, so distinct are these two phases. Cohen and Clark [3] found the optimum P_H for the two phases might be different, and the recent work of Sherman and Albus [4] shows that 24-hour cultures "have settled down to a more or less inactive state so far as reproduction is concerned." Berghaus[5] in Rubner's laboratory, unsuccessfully attempted to differentiate the two phases by determinations of ammonia production. Kendall and his co-workers have published extensive evidence supporting the theory of such diphasic character of bacterial metabolism, and have translated their chemical findings into terms of structural and energy food utilization. Kendall's [6] early work demonstrated the protein-sparing power of carbohydrates in bacterial metabolism. He further divided products of bacterial activity into endogenous and exogenous origin,[7] finding enzymes responsible for the latter and ammonia production a measure of the former, when protein is used for energy.

Experimental

Mediums and Inoculums.—A medium which would support excellent growth and toxin production, and one from which uniform samples could be removed, was desired. A mixture of equal parts of double strength veal infusion and peptic digest made according to the method of Stickel and Meyer,[8] was believed to answer these requirements. Enough 25% sterile glucose solution was added to one set of the flasks to make a final concentration which chemical tests showed to be 1.32%. It was planned to make the experiments in duplicate, and accordingly four 2-liter Erlenmeyer flasks, each containing 1,800 c c. of medium, were set up for inoculation and incubation at 37 C. in the water bath to be described. Each flask received 20 c c. of a spore suspension of strain 38 B. botulinus just previously heated for 1 hour at 80 C. Direct counts by means of a Helber chamber had revealed approximately 1,000,000,000 spores per c c. of suspension. Methods of evacuation and sampling will be given in detail in another paper. Unfortunately, one flask collapsed under the reduced pressure, hence duplicate determinations were possible with the glucose cultures only, and since the results of the latter were remarkably similar only one set of determinations is reported. The flasks were always well rotated just previous to sampling to accelerate the liberation of gases and to mix the cultures thoroughly. At the termination of the experiment, approximately one-half of the medium remained, and the total nitrogen content had increased slightly.

[3] Jour. Bacteriol., 1919, 4, p. 409.
[4] Ibid., 1923, 8, p. 127.
[5] Arch. f. Hyg., 1908, 64, p. 1.
[6] Jour. Med. Res., 1911, 25, p. 117.
[7] Jour. Infect. Dis., 1915, 17, p. 442.
[8] Ibid., 1918, 23, p. 68.

Samples removed late at night were held in the icebox until the morning following, but all other determinations were made promptly.

Plating and Anaerobic Incubation Methods.—The 0.5% glucose-peptic digest-beef heart infusion used for dilutions, and the 0.5% glucose-peptic digest-beef heart infusion-sheep blood agar used for plating have been described in detail in another communication, as have improved anaerobe jars[9] which insured dependable anaerobiosis. A description and illustration of characteristic colony form on this medium is included in the preceding paper of this series.[10]

TABLE 1

GROWTH OF B. BOTULINUS STRAIN 38 ON VEAL INFUSION-PEPTIC DIGEST WITH AND WITHOUT GLUCOSE

	Time, Hrs.	Total N, Mg.	Amino N, Mg.	Ammonia N, Mg.	Volatile Acid N/10, C c.	P_H "C"	Sugar, %	Gas per Liter, C c.	Creatinin, Mg.	CO_2, C c.	M. L. D. for Mice, C c.	Organisms per C c.
"A" Glucose-Veal Infusion Peptic Digest	0	614	160	33.0	15.6	6.81*	1.32	.....	17.5	3.9	0	1,850
	12	...	160	35.0	....	.....	1.32	75	17.9	....	0	5,000
	18	...	149	49.0	....	6.8	....	132	....	....	0.1	95,000,000
	21	...	174	87.0	27.6	6.84*	....	209	....	....	.....	545,000,000
	24	...	195	127.0	40.0	.....	1.21	398	....	38.5	0.001	760,000,000
	27	...	201	141.0	56.0	6.6	....	518	....	....	.....	149,750,000
	30	...	...	153.0	....	6.6	....	751	15.7	64.3	0.0002	1,0,750,000
	36	...	231	156.0	....	6.6	1.10	965	19.2	....	.....	49,000,000
	42	...	...	.....	62.0	6.7	0.81	1,165	19.6	....	.....	11,975,000
	48	...	211	156.0	....	...	....	1,590	....	64.0	0.0001	15,950,000
	60	...	204	136.0	....	6.5	0.41	1,990	14.9	....	0.000002	6,625,000
	72	...	245	136.0	70.0	6.5	0.17	2,384	13.1	....	0.00001	18,325,000
	80	...	...	.....	....	...	0.0	2,830	....	....	.....	.....
	96	...	249	105.0	78.0	...	....	2,920	18.3	14.2	0.00001	520,000
"B" Veal Infusion Peptic Digest	0	629	165	36.6	15.0	7.0	....	.....	17.5	3.9	0	1,000
	12	...	...	.....	....	...	....	58	....	....	.....	.....
	18	...	165	40.3	....	6.8	....	116	....	....	0	18,700,000
	21	...	...	.....	....	...	....	.....	....	....	.....	.....
	24	...	187	56.4	38.5	6.7	....	358	....	....	0.01	236,500,000
	27	...	171	146.0	44.0	6.6	....	490	....	....	.....	.....
	30	...	...	180.0	....	6.6	....	634	16.5	93.1	0.005	43,000,000
	36	...	180	173.0	48.8	6.7	....	880	....	....	0.00001	6,360,000
	42	...	186	.....	....	...	....	1,090	14.3	....	0.000001	1,870,000
	48	...	187	190.0	70.8	...	....	1,375	....	80.0	0.00001	727,500
	60	...	183	192.0	....	6.8	....	1,640	18.8	70.0	0.00001	1,270,000
	72	...	194	204.0	....	6.8	....	.....	16.5	....	.....	5,300,000
	80	...	...	.....	....	...	....	1,870	....	....	.....	.....
	96	...	226	218.0	73.2	...	....	1,920	18.4	78.0	.....	.....

* Determination made electrometrically.

Frequency of Determinations.—Samples from which chemical determinations and plate counts were made were removed immediately after planting. Weil[11] found it took 8 hours at 30-37 C. for the majority of anthrax spores to germinate, and the time required for germination of the majority of B. botulinus spores is probably not very different. The second set of determinations was therefore made on the 12th hour, by which time it was thought slight multiplication of the organisms would have occurred with resulting chemical changes in the medium. The third set of analyses was made on the 18th hour, after which, up to and including the 30th hour, 3-hour intervals only were allowed between samplings.

[9] Ibid., 1922, 31, p. 617.
[10] Ibid., 1923, 33 p. 377.
[11] Arch. f. Hyg., 1889, 35, p. 355.

Three 6-hour intervals elapsed, and then longer times intervened. During the period of intensive sampling it was anticipated that plate counts and chemical determinations showing the amount of utilization of sugar and ammonia accumulation would yield information regarding structural and energy phases of the metabolism of B. botulinus.

CHEMICAL CHANGES PRODUCED BY B. BOTULINUS

Sugar Consumption.—Sugar determinations were made by the Shaffer and Hartman method,[12] which permits the measurement of

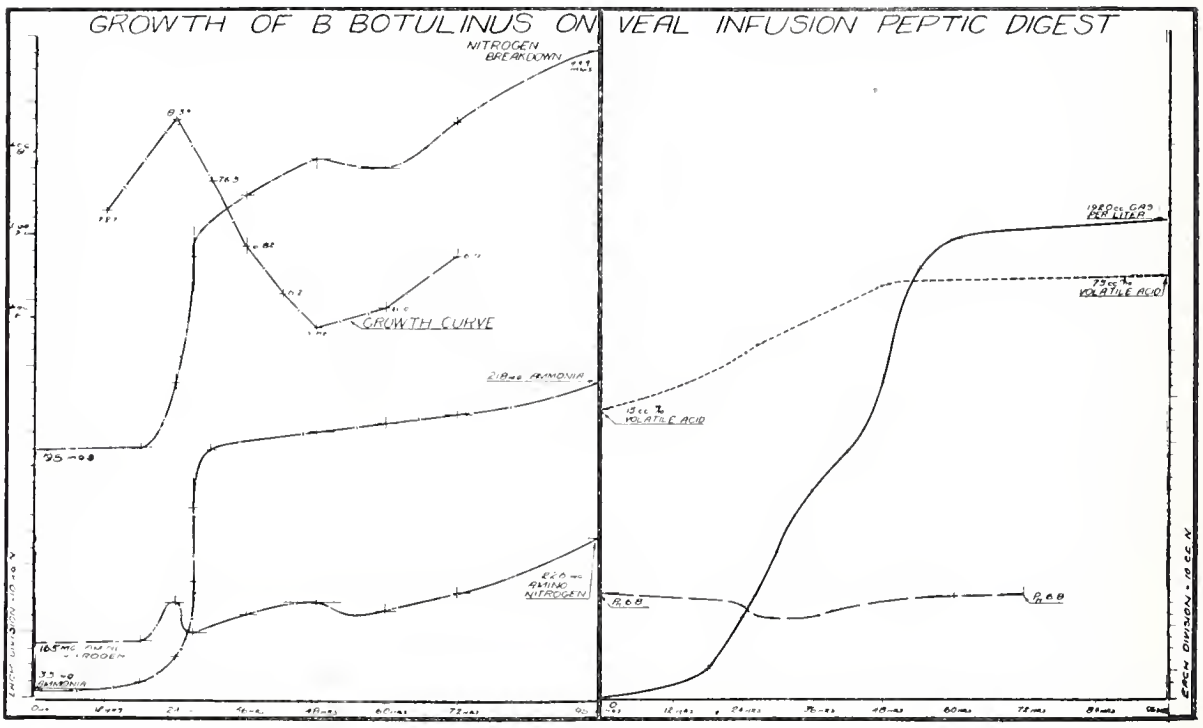

Chart 1

much smaller amounts of glucose than the method used in previous experiments. Filtrates from the tungstic acid precipitation were used for sugar determinations. In such a dilution the effect of creatinin and minute amounts of other interfering substances was negligible, and the acid products of fermentation did not interfere with iodometric titrations.

Initial determinations showed the presence in culture A of 1320 mg. of glucose per 100 c c. of medium. Further analyses showed utilization of this sugar as follows: first 24 hours of incubation, 110 mg.; next

[12] Jour. Biol. Chem., 1920, 45, p. 365.

12 hours of incubation, 110 mg.; next 6 hours of incubation, 285 mg.; next 18 hours of incubation, 406 mg.; next 12 hours of incubation, 230 mg., and next 12 hours of incubation, 170 mg. (sugar exhausted).

As has already been noted, determinations on the duplicate sugar containing culture were almost similar, and are of particular interest in that they also showed the slow utilization of glucose during the first 24 hours of incubation. No account has been taken of the small amount of muscle sugar which was undoubtedly present in culture B, and which is included in the 1.32% of sugar of culture A.

Gas Production.—The gas was measured over water, as will be described in a later paper. While this procedure is not quantitatively

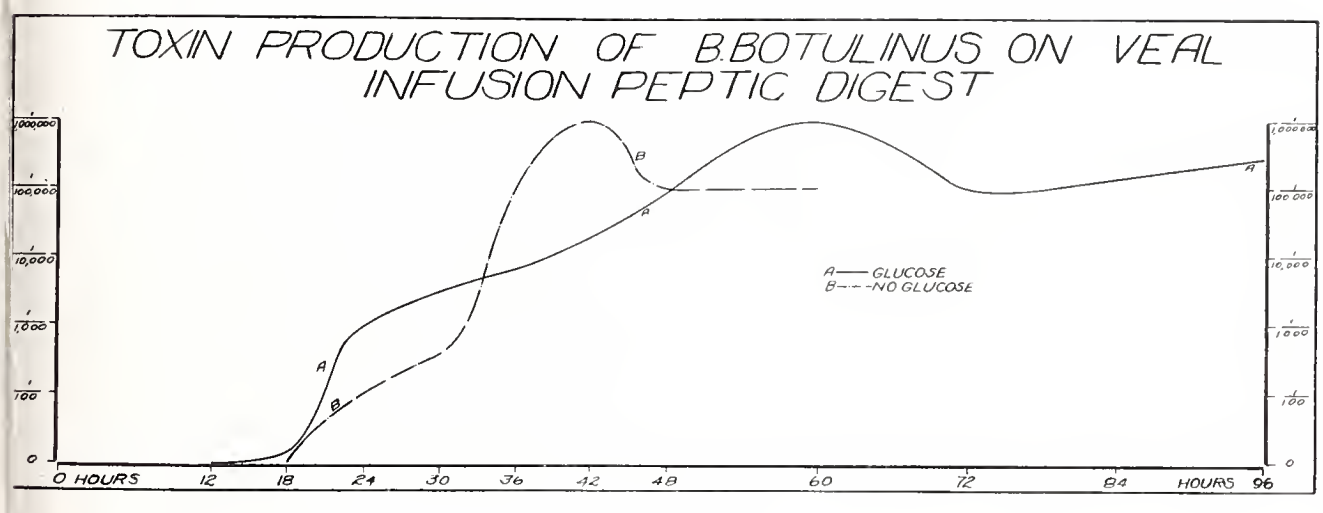

Chart 2

accurate, because of solubility of some of the gases, it permits of comparative results. Gas was produced in both cultures by the 12th hour, and accumulated slowly up to the 18th hour. The rate of production then increased markedly in both cultures. In A, the gas was formed at a more or less regular rate from the 18th to the 80th hour, while in B the formation was fairly regular from the 18th to the 60th hour, when the production fell off appreciably. The data for the first 60 hours, when charted, gave rise to remarkably similar curves (charts 1 and 2). However, at the end of 96 hours, 1,000 c c. more gas had been formed by glucose culture A than nonglucose culture B.

Reaction of Cultures.—The hydrogen-ion concentration of culture B increased slowly from a P_H of 7.0 until 6.6 was reached on the 27th hour. A reversion to P_H 6.8 was noted on the 60th hour. The reac-

tion in A during the course of the experiment changed from P_H 6.81 to 6.5. A greater change in reaction usually occurs in mediums containing as much as 1.32% of utilizable carbohydrate. Buffer index determinations offer an explanation.

Buffer Index Determinations.—Results of titrations by the Brown [13] method for the buffer content of the cultures are shown in table 2. Seventy-seven c c. of N/10 acid were required to change the reaction of 100 c c. of the medium to P_H 5, and 40 c c. of N/10 alkali to bring the reaction to P_H 8. The richness of this medium in buffer material (buffer index 11.7) as compared with the veal broth used by Brown (buffer index 4),[13] or the 2% Difco peptone solutions to be discussed in another paper (buffer index 1.5) accounts for the slight increase in the hydrogen-ion concentration in culture A. The fermentation of the 1.32% sugar present resulted in a decrease of the buffer index on the acid side from 7.7 to 6.3 during the first 48 hours, but the culture had accumulated enough acids during that time to increase the buffer index on the alkaline side from 4 to 7. During the next 48 hours the acids produced depressed the buffer index on the acid side from 6.3 to 2.2, and it may be predicted that had more sugar been present further fermentation would soon have made a distinct change in the P_H of the medium. The reserve alkali of culture B increased until it required more than a half more acid to bring the reaction to P_H 5 than it had in the original culture medium.

TABLE 2

Buffer Determinations

	N/10 NaOH to P_H 8, C c.	N/10 H_2SO_4 to P_H 5, C c.	Buffer Index
Control medium, 10 c c.	4.6	7.7	11.7
Culture (A) 48 hours, 10 c c.	7.0	6.3	13.3
Culture (A) 96 hours, 10 c c.	7.1	2.2	9.3
Culture (B) 60 hours, 10 c c.	2.0	12.3	14.3

Volatile Acids.—The volatile acids accumulated rapidly in both cultures during the first 27 hours, after which the rate of production decreased abruptly in culture A, while in B the accumulation continued at approximately the same rate until after the 48th hour. Determinations on the 96th hour showed the two cultures had produced almost similar amounts of volatile acids.

[13] Jour. Bacteriol., 1921, 6, p. 555.

The acids were recovered from the samples of the cultures by steam distillation and identified by the fractionation procedure of Duclaux, as given by Bertrand and Thomas.[14] Too many distillations are not advisable on account of the inevitable losses. Accordingly, the distillate was collected in two fractions. Each of these was again distilled, and the 10 c c. fractions collected and titrated with NaOH. An idea of the acids present was secured by comparing the sequences of these titration figures with the titration figures of similar distillates from

TABLE 3

VOLATILE ACID PRODUCED BY B. BOTULINUS ON VEAL INFUSION-PEPTIC DIGEST WITH AND WITHOUT GLUCOSE

	"A"		"B"	
	96 Hour Culture		96 Hour Culture	
	1st Fraction	2d Fraction	1st Fraction	2d Fraction
C c. N/10 sodium hydroxide to neutralize volatile acids	26.9	7.0	20.7	11.3
Percentage of volatile acid in fraction of distillate (Duclaux method) — 10 c c.	22.6	14.3	23.2	13.3
20 c c.	39.8	25.7	41.0	18.6
30 c c.	54.0	37.2	55.5	26.6
40 c c.	65.5	47.2	66.0	35.4
50 c c.	75.0	55.8	75.0	44.2
60 c c.	82.6	64.4	81.6	54.0
70 c c.	88.5	73.0	87.0	63.6
80 c c.	93.3	81.5	92.0	74.5
90 c c.	97.0	90.0	95.7	86.5
100 c c.	100.0	100.0	100.0	100.0
Ratio of acids present from titration sequence	3 valeric, 1 acetic	1 butyric, 1 acetic	3 valeric, 1 acetic	1 butyric, 4 acetic
	Gm.	Gm.	Gm.	Gm.
Volatile acids as sodium salts found	0.3258	0.0773	0.2875	0.1403
Calculated as sodium salts from titration value	0.3406	0.0861	0.2627	0.1477
Difference between volatile acid sodium salts found and calculated	0.0148	0.008	0.0248	0.0074

solutions of known acids. Additional information concerning the identity of the acids was secured by sodium salt computations. After the titration of the volatile acids the sodium salts were dried and weighed. The weight of the sodium which neutralized the volatile acids was easily computed. This amount of sodium in combination with volatile acid radicals in the proportion indicated by the titration figures would give a weight of salt which agrees closely with the weights of salts actually found. This agreement is further proof that the acids in both cultures were valeric, butyric and acetic. Culture A contained these in the approximate ratio of 7 : 1 : 3, and culture B, 5 : 1 : 4, respectively.

[14] Practical Biological Chemistry. 1921, p. 293.

Nonvolatile Acids.—Concentrates of the fixed acid solutions remaining in the steam distillation flasks after removal of the volatile acids were extracted for 72 hours in a modified Soxhlet apparatus. The ether was distilled off and water solutions of the acids titrated with an excess of $Ba\ OH_2$. This excess was titrated with N/10 H_2SO_4. These solutions were filtered and the filtrates containing the barium salts of the fixed acids were evaporated, weighed and the salts converted into barium sulphate and again weighed. The weight of barium lactate which could be formed from the barium portion of the barium sulphate was calculated. These data are listed in table 4. The figures thus secured correspond quite closely to the weights of salts actually found, and are in closer agreement than are the calculated weights of the salts of various other organic fixed acids which might be present.

TABLE 4

NONVOLATILE ACIDS

	N/10 Nonvolatile Acid 100 C c. of Culture, C c.	Nonvolatile Acids of Aliquot Part as Barium Salt, Gm.	Barium Sulphate Equivalent		N/10 Lactic Acid per 100 C c. Culture Calculated from $BaSO_4$, C c.
			Found, Gm.	Calculated for Lactic Acid, Gm.	
Culture A 96 hours.....	23.2	0.2173	0.1383	0.1062	23.6
Culture B 96 hours.....	32.8	0.208	0.1345	0.1045	29.0

Positive Uffleman's tests also indicated the presence of lactic acid. The difference in the calculated and actual weights of sodium lactate suggest the presence of small amounts of unidentified fixed acids. Culture A contained 23.2 c c. of N/10 fixed acids; culture B 32.8 c c.

Carbon Dioxide.—The free and combined carbon dioxide in the cultures was determined with the Van Slyke apparatus.[15] One c c. amounts were analyzed. Three and nine tenths c c. of carbon dioxide were liberated from 100 c c. of medium by dilute sulphuric acid. The carbon dioxide increased until on the 30th hour A contained 64.3 c c. and B, 96.1 c c. As incubation proceeded the acidity developing in A, as shown by the buffer index, caused carbon dioxide to be expelled. On the 96th hour there were 24.2 c c. in A and 78 c c. in B per 100 c c. of culture.

Nitrogen Changes.—(1) Total Nitrogen: The initial total nitrogen contents of cultures A and B were 614 and 629 mg. per 100 c c. of

[15] Jour. Biol. Chem., 1917, 30, p. 289.

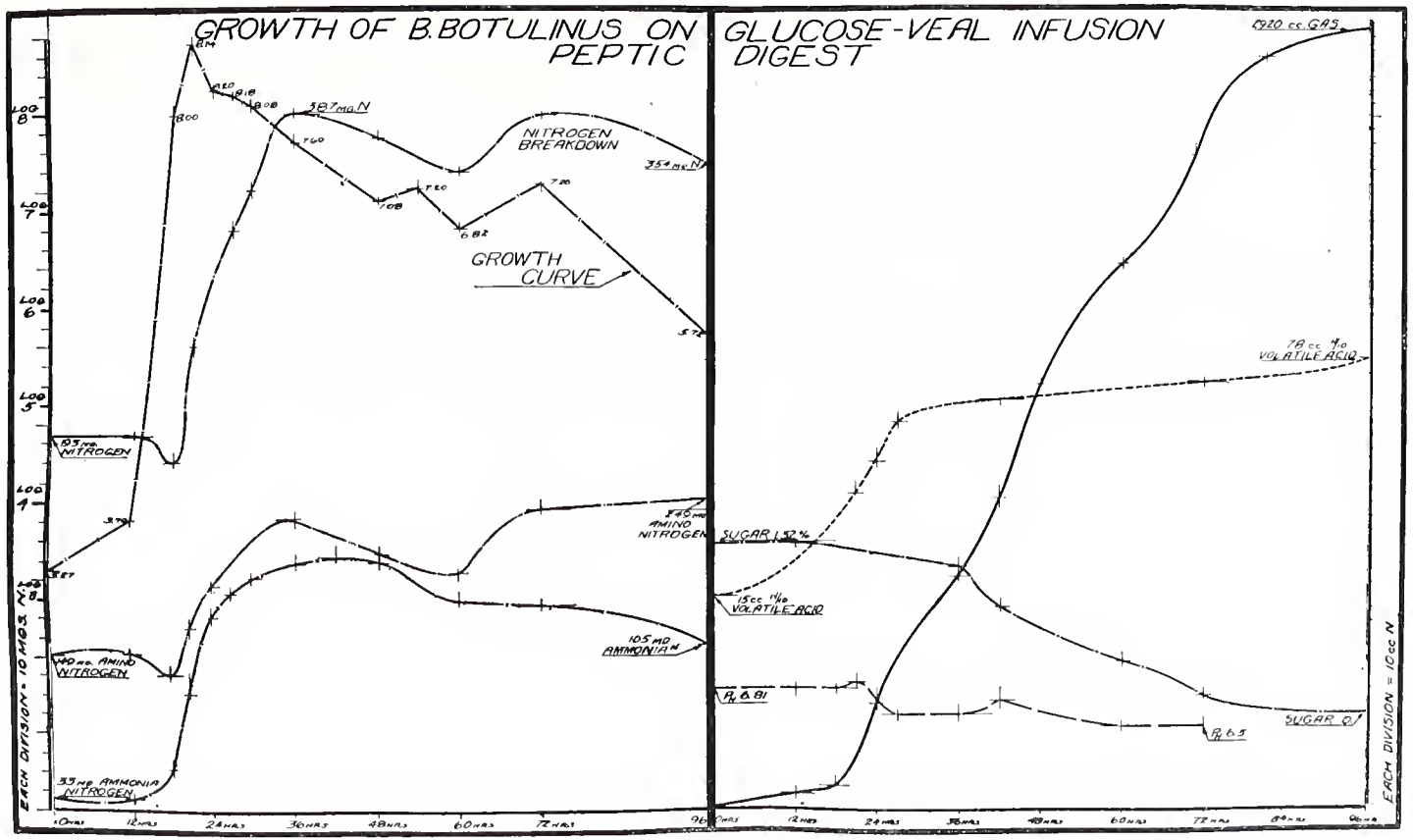
GROWTH OF B. BOTULINUS ON PEPTIC
GLUCOSE-VEAL INFUSION DIGEST
NITROGEN BREAKDOWN
GROWTH CURVE
387 mg N
354 mg N
103 mg NITROGEN
140 mg. AMINO NITROGEN
33 mg AMMONIA NITROGEN
546 mg AMINO NITROGEN
105 mg AMMONIA N
920 cc GAS
78 cc % VOLATILE ACID
15 cc % VOLATILE ACID
SUGAR 1.52%
SUGAR 0
Ph 6.81
Ph 6.5
EACH DIVISION = 10 MG. N
EACH DIVISION = 10 cc N
LOG 8
LOG 7
LOG 6
LOG 5
LOG 4
LOG 3
10 HRS
12 HRS
24 HRS
36 HRS
48 HRS
60 HRS
72 HRS
96 HRS

Chart 3

medium, respectively. After 12 days of incubation the amounts were 623 mg. for A and 658 mg. for B, which indicate negligible increases.

(2) Amino Acid Nitrogen: The amino acid nitrogen of cultures A and B increased during the first 96 hours of incubation from 160 and 165 mg. per 100 c c. to 249 and 226 mg., respectively. Fluctuations occurred in both cultures during this time, as may be noted from figures in table 1 or by graphic representations in charts 1 and 2.

(3) Ammonia Nitrogen: Ammonia began to accumulate rapidly in culture A after the 18th hour, and by the 36th hour the ammonia nitrogen had increased from 33 to 156 mg. per 100 c c. of medium. This concentration was never exceeded, and on the 96th hour had decreased to 105 mg. The beginning of rapid accumulation of ammonia nitrogen in culture B was delayed until after the 24th hour. An increase from 56.4 to 180 mg. per 100 c c. occurred between the 24th and 30th hours, and on the 96th hour this amount had further increased to 218 mg. per 100 c c. These data are shown graphically in charts 1 and 2.

Toxin Tests.—Tests for toxicity were made on white mice by intraperitoneal inoculation of 1 c c. amounts of successive dilutions of the supernatant fluid secured by centrifugation at high speed for one hour. The MLD was the amount of broth diluted with phosphate saline which caused death in approximately 96 hours. The MLD of culture A on the 18th hour was 0.1 c c. The potency of the toxin increased rapidly until it reached a maximum of 0.000002 c c. on the 60th hour. A slight loss in toxicity may have occurred by the 72nd hour, and on the 96th hour the MLD was the same, 0.00001 c c. No toxin could be demonstrated in culture B on the 18th hour, but on the 24th hour the MLD was 0.01 c c. The potency had increased to 0.000001 c c. on the 42nd hour, and on the 48th hour a decrease to 0.00001 c c. had occurred, which titer had not changed on the 60th hour.

DISCUSSION

A comprehensive view of the metabolic activities of B. botulinus in the two mediums is afforded by the curves of growth, sugar utilization, ammonia production and amino acid formation, together with a curve representing the total nitrogen of the last two substances which for want of a better appellation is designated "nitrogen break-down."

The curves of growth, particularly the curve of culture A, attest to the high biologic value of equal parts of double strength veal infusion-peptic digest for B. botulinus. The number of organisms reaches a higher level, which is maintained for a longer period in the

medium containing glucose. Plate counts show that the peak of proliferation occurred in both cultures around the 24th hour, but it could not be expected that this would mark the abrupt end of the structural phase, since some proliferation undoubtedly occurred after that. Neither could it be hoped to determine the beginning of the energy phase by detection of a complete change in biochemical activities, since the energy phase of some of the organisms would begin early in the incubation period and there would be a gradual assumption of that phase by the culture as a whole. The continual interplay of the two phases is shown by the plate counts, and indicated by the uneven curves representing biochemical activities. The rather abrupt break in the curve of sugar consumption on the 36th hour is probably more apparent than real, since 9 hours had elapsed since the previous determination. If the liberty is taken, however, of making the 24th hour of incubation a dividing line, structural and energy phases can easily be distinguished. During the first 24 hours the sugar consumption in culture A was 110 mg. per 100 c c. of culture, while during the following 48 hours 1040 mg. were consumed, or an hourly rate of consumption approximately five times as high as it had been during the structural phase.

The separation of the two phases in culture B is even more distinct. The ammonia accumulation during the first 24 hours was about 20 mg. per 100 c c. while during the ensuing 48 hours 133 mg. accumulated, or rates of accumulation represented by a ratio of 1 to 3. During the interval from the 24th to the 27th hour, a period which from the plate counts might be anticipated would cover the maximum hourly energy need, 90 mg. of ammonia accumulated. In other words, the hourly rate of the energy metabolism as measured by the accumulation of ammonia, during the 3 hours immediately succeeding the period of maximum number of viable organisms, was 35 times as high as during the 24 hours of the structural period. This is a striking confirmation of Kendall's [16] conclusion: "The energy requirements of bacterial cells are as disproportionately extensive as the structural requirements are inconspicuous."

Culture A determinations on the 12th hour are extremely interesting. The sugar had not been attacked, yet plate counts showed reproduction had occurred, and biochemical activity was shown by the formation of not inconsiderable quantities of gas. While the amino acid nitrogen had not increased in amount, it was probably being formed and used at equal rates. Experimental evidence is reported in another paper [10]

[16] Jour. Infect. Dis., 1922, 30, p. 211.

showing the tendency of B. botulinus to degrade complex nitrogen compounds in the presence of large amounts of amino acids. It seems likely that reproduction was proceeding at the expense of amino acids, and it is highly probable that a selective action was in progress. The richness of bacterial protein in basic amino acids is, of course, universally accepted. One extreme of this view is held by Vaughan,[17] who remarks: "Bacteria may be more properly defined as nuclei, probably protected by a protein ectoplasm. . . . Chemically, bacteria are nucleoproteins." Long[18] found nucleic acid made up 2 to 3.5% of the dry weight of tubercle bacilli. It may be presumed that even though the amino acids richest in nitrogen were selected for bacterial protein, the nitrogen groups were being dislodged from some acids and concentrated in others prior to assimilation. Such a hypothesis affords a basis for an explanation of gas formation and lack of sugar utilization during the first 12 to 18 hours of incubation. Hhe deaminized portion of the amino acid molecules not used for structural purposes would supply, under these conditions, the energy needs of the organisms, thus reversing the usual process and "sparing" the carbohydrate.

It would naturally be expected that immediately following the maximum growth period the energy requirements would be at the highest. In A the sugar was used only sparingly between the 18th and 36th hours, and the ammonia nitrogen was formed at a rate similar to the rate in B. The only conclusion which seems permissible is that amino acids chiefly were used during this time for energy. It should perhaps be repeated that determinations on a duplicate sugar-containing culture show this same phenomenon. It is worthy of mention, though, that the amino acid level after the 24th hour of incubation was higher throughout the 4 days of the experiment in culture A, which is indicative of a sparing action of the sugar on the protein, proteose or polypeptid constituents of the medium. It may be noted in this connection that Sears[19] concluded the protein-sparing action of carbohydrates is greater for aerobes than anaerobes.

During the period from the 36th to the 48th hours, which marked the height of the energy phase if the amounts of sugar utilized is a measure, the ammonia accumulation ceased. During the period immediately following this, however, there was a loss in ammonia nitrogen, the reasons for which are not evident. Ammonia is extremely soluble

[17] Infection and Immunity. Commemoration Volume, Am. Med. Assn., 1915, p. 18.
[18] Am. Rev. Tuberc., 1921, 4, p. 842.
[19] Jour. Infect. Dis., 1916, 19, p. 105.

and as the reaction remained acid no loss could be expected through volatilization. Quantitative measurements to determine the possibility of volatilization of ammonia from bacterial cultures have been made by Bosworth, Elkins and Blanchard,[20] who concluded: "So long as the medium remains acid both total and ammonia nitrogens show no decrease." The methods employed for the liberation of ammonia have been subjected to critical study, and some of the results reported.[10] It may be stated though that there is no indication that sodium carbonate fails to give consistent results. The most probable explanation for the decrease in ammonia nitrogen seems to be that of synthesis.

Bierema [21] and Doryland [22] showed that ammonia in the presence of a utilizable carbohydrate might be consumed even by ammonifying organisms. Sears' [19] results led him to conclude that most species of bacteria "show evidences of a continuous utilization of ammonium salts in small amounts." Waksman [23] suggests that "Ammonia which may be only a waste product for some organisms may act as an intermediate product for others." Long [24] working it is true with acid-fast bacilli, which grow with comparative ease on synthetic mediums, is inclined to believe that ammonia rather than amino acids is the starting point for intracellular synthesis. Liot [25] concluded that ammonium salts of certain acids are sufficient to enable B. pyocyaneous to form its characteristic pigment, and furthermore, if an ammonium salt is not provided the organism evolves one from the constituents of its nutritive environment before such a growth product appears. While B. botulinus has never been cultivated on inorganic nitrogen solely, it is not at all impossible that small quantities from such sources are continuously used, but owing to rapid accumulation of ammonium compounds in the culture mediums such utilization is generally masked. And it is not impossible that if optimum carbon nutritional needs, which may be as specific as is the need of tubercle bacilli for glycerol, could be supplied, inorganic nitrogen might be found to support growth and reproduction. This question will be discussed more in detail in a subsequent paper. The possible necessity of accessory substances in the nutrition of B. botulinus will likewise be discussed in the same paper, but it may be mentioned that the vitamines generally concerned in animal nutrition apparently play no part in its metabolism.

[20] Ibid., 1922, 30, p. 357.
[21] Centralbl. f. Bakteriol., II, 1909, 23, p. 672.
[22] N. D. Agric. Experi. Sta. Bull., 116, p. 916.
[23] Jour. Bacteriol., 1918, 3, p. 475.
[24] Am. Rev. Tuberc., 1919, 3, p. 86.
[25] Ann. de l'Inst. Pasteur, 1923, 37, p. 234.

On the 96th hour culture B contained 113 more mg. of ammonia nitrogen than culture A, which on the whole seems to be the best measure of the sparing power of sugar on the utilization of nitrogenous compounds for energy. Ammonia determinations on the 12th day of incubation (not included in table 1) indicated negligible metabolic activity during the preceding 8 days.

No doubt exists that the amount of sugar which can be utilized by bacteria depends to a large extent on the buffer content of the medium. Initial buffer index determinations indicated a marked ability of this medium to resist changes in reaction, and no appreciable increase in the hydrogen-ion concentration resulted from the metabolism of approximately twice the amount of sugar that has caused an inhibitive reaction in a 2% peptone solution. It may be surmised that more sugar would have been utilized had it been present.

Nitrogen compounds intermediary between amino acid and ammonia undoubtedly exist in bacterial cultures. Among those most often looked for are indol and amines. The former has frequently served as a means of differentiating organisms, but Norton and Sawyer [26] warn against its use as an absolute criterion. They are as uncertain of its significance as were Tissier and Martelly [27] in 1902. Homer [28] and Logie [29] believe tryptophane necessary for bacterial growth. With an abundance of that amino acid in the medium there is splitting off of indol, but if there is a lack indol may be used for the synthesis of tryptophane. They found glucose in the medium increased the demand for indol due to more rapid reproduction.

Tests by the Gou [30] method failed to show the presence of indol in either culture A or B. Negative tests for indol in B. botulinus cultures have been reported by Norton and Sawyer [26] Hall [31] and Oman and Meyer [32] Kendall [33] states that obligate anaerobes do not form indol.

Amines, more commonly called ptomaines, are bases formed by the destruction of amino acids through decarboxilation. Their production is influenced, according to Wells,[34] by the nature of the bacteria, the composition of the medium, the temperature and duration of incubation,

[26] Jour. Bacteriol., 1921, 6, p. 471.
[27] Ann. de l'Inst. Pasteur, 1902, 16, p. 865.
[28] Jour. Hyg., 1916, 15, p. 401.
[29] Jour. Path. and Bacteriol., 1920, 23, p. 224.
[30] Indian Jour. Med. Res., 1921, 8, p. 505.
[31] Jour. Infect. Dis., 1922, 30, p. 445.
[32] To be reported.
[33] Endocrinology and Metabolism, 1922, 3, p. 670.
[34] Chemical Pathology, 1920, p. 117.

and the quantity of oxygen available. Many of those of higher molecular weight are toxic to animal tissue, but are in no way related to the true toxins. Ellinger [35] demonstrated that anaerobic conditions favor the production of amines, and since they contain little or no oxygen, this result is not unexpected. Herter and Broeck [36] identified primary amines in a glucose-free meat extract-casein-peptone culture of B. proteus vulgaris. Bertrand and Berthelot [37] isolated from intestinal contents an organism which in pure culture, in the absence of glucose, formed amines from histidine, tryosine and tryptophane. Schubel [38] identified trimethylamine in cultures of B. botulinus.

The method of Weber and Wilson,[39] which provides for the detection and identification of volatile amines in the presence of ammonia, was used to test cultures A and B at various periods of incubation. The original medium contained 14 mg. of mono-amine nitrogen per 100 c c. No change in the amounts could be detected in either culture on the 27th and 60th hours, but on the 96th hour 22 and 18 mg., respectively, were found. These results suggest that only minute quantities of the comparatively large amounts of amino acids present, if any, were decarboxylized by B. botulinus, and indicate that the effect of glucose was negligible, and also that the course of breakdown of amino acids by B. botulinus is not generally by way of amines. Long [40] has recently concluded that the katabolism of amino acids by the tubercle bacillus is not by this route.

Considerable interest has been attached to creatinin production and utilization by bacteria. An unsuccessful attempt to differentiate the various members of the mucosus capsulatus group of bacteria was made by Schmidt and Fitzgerald [41] on the basis of creatinin production. B. proteus vulgaris was the only organism they tested which formed measurable amounts of this compound in 2% Witte's peptone solutions. Sears,[19, 42] however, found that a considerable number of bacteria was able to form creatinin in 2% peptone solutions when glucose was added. He explains the phenomenon as a sparing effect of the sugar for creatinin.

[35] Ztschr. f. physiol. chem., 1900, 29, p. 344.
[36] Jour. Biol. Chem., 1911, 9, p. 491.
[37] Lancet, 1913, 84, p. 523.
[38] Deutsch. med. Wchnschr., 1921, 47, II, p. 1047.
[39] Jour. Biol. Chem., 1918, 35, p. 385.
[40] Am. Rev. of Tuberc., 1921, 5, p. 705.
[41] Proc. Soc. Exper. Biol. & Med., 1912, 10, p. 55.
[42] Jour. Bacteriol., 1917, 2, p. 187.

Determinations for creatinin were made by the Folin [43] colorimetric method. The medium contained 17.5 mg. of creatinin per 100 c c. While the results reveal some variation in the amounts present at different times, they indicate that the bacteria were neither attacking nor forming this compound to any great extent. There was no indication that the sugar exerted any favorable reaction on the production of creatinin.

The similarity of the toxin titers of the two cultures is not unexpected. Experimental evidence which supports the hypothesis that the matrix of the poison is the bacterial cell from which the toxin is liberated during autolysis has accumulated in this laboratory. Several lines of evidence supporting this theory can be traced through the preceding experiments. In the first place, toxin was present in glucose-containing culture A before it could be detected in culture B and growth, and it may be expected, death and consequent autolysis of some of the organisms occurred first in the former. While plate counts showed a greater number of viable organisms in A, the death rate in B was accelerated and therefore it is not unlikely that autolysis proceeded more rapidly, accounting for the similarity of the toxin titers of the two cultures.

Another source of evidence substantiating this theory is the fact that the production of the poison must be different from that of B. diphtheriae or of B. tetani, whose toxins Kendall [16] considers striking examples of by-products formed during the forced utilization of protein for energy. There is considerable evidence in the literature suggesting the exogenous source of these toxins in contrast to the apparently endogenous origin of B. botulinus toxin. Theobald Smith [44] first recognized the "sparing" action of glucose on the toxin production of B. diphtheriae, a relationship which has not been found for B. botulinus. Davis [45] could not correlate vigor or growth and toxin production by B. diphtheriae. Robinson and Rettger [46] found beef infusion favored growth and peptones toxin production in cultures of that organism. Their work did not confirm that of Uschinsky [47] nor Hadley,[48] who claimed toxin was produced by synthesis from inorganic nitrogen compounds, but was in harmony with the results of Hida [49] and Dziergowski

[43] Jour. Biol. Chem., 1919, 38, p. 81.
[44] Jour. Exper. Med., 1899, 4, p. 373.
[45] Jour. Lab. and Clin. Med., 1917, 3, p. 75.
[46] Jour. Med. Res., 1917, 36, p. 357.
[47] Centralbl. f. Bakteriol., 1897, 21, p. 146.
[48] Jour. Infect. Dis., 1907, Suppl. 3, p. 95.
[49] Ztschr. f. Hyg., 1908, 61, p. 273.

and Rekowski,[50] who concluded the toxin was formed from proteoses or higher polypeptids of the medium, and those of McConkey,[51] who found a seasonal variation in the production of toxin. No confirmation of Warden, Connell and Holly's [52] work on the nature of toxins has appeared. They believe that toxin is a complex of which unsaturated fatty acids are the chief component, and that diphtheria toxin is a disintegration product of the organism.

Dubovsky and Meyer [53] found that a van Ermengem medium, containing 2% glucose, which formerly was considered optimum for the growth of B. botulinus, does produce potent toxins and fair growth of pure strains of the organisms. Their results are evidence of the failure of sporulation. The majority of the bacilli in such a medium are undoubtedly short lived, and it may be suspected the production of toxin is favored thereby. Schubel [38] considered a medium containing 2.5% of glucose optimum for the production of toxin.

SUMMARY AND CONCLUSIONS

The metabolism of B. botulinus in veal infusion-peptic digest and in a similar medium 1.32% of glucose has been studied by means of various determinations at short successive intervals during the first 96 hours of incubation. The analyses included: (1) the number of viable organisms by plate counts, (2) the composition of the culture mediums by chemical tests, and (3) the toxic titers of the two cultures by biologic tests. From the results of these parallel triplicate series of determinations the following conclusions may be drawn:

The presence of glucose in a nitrogen-rich, well buffered medium increased its biologic value for B. botulinus. It stimulated early reproduction, the maximum number of viable organisms was greater, and a higher level of growth was maintained throughout the experimental period.

The presence of glucose exerted little effect on the accumulation of ammonia before the 36th hour, after which there was a gradual loss of ammonia in contrast to a continuous increase in the glucose-free culture. The suggestion has been made that in the presence of glucose synthesis occurred. A sharp increase in the accumulation of ammonia occurred in the glucose-free culture coincidently with the presence of the greatest number of viable organisms.

[50] Arch. d. sc. biol., 1892, 1, p. 167; cited by Robinson and Rettger.[18]
[51] Jour. Hyg., 1912, 12, p. 507.
[52] Jour. Bacteriol., 1921, 6, p. 103.
[53] Jour. Infect. Dis., 1922, 31, p. 501.

There was, on the whole, a gradual increase in amino acid nitrogen in both cultures, but the total amount on the 96th hour was greater in the medium containing glucose.

Only small amounts of sugar disappeared prior to the 24th hour, and the period of greatest sugar utilization was from the 36th to the 48th hour, a period which was not coincident with the highest level of growth, as shown by plate counts. These results do not entirely support the conception that fermentation always takes preference over putrefaction.

No sharp line of division between the structural and energy phases could be distinguished in the presence of glucose, but in the glucose-free culture a sudden marked increase in the accumulation of ammonia occurred coincidently with the greatest number of viable organisms.

Glucose had little influence on the total amount of volatile acids produced. Valeric, butyric and acetic acids were present in both cultures, but in somewhat different proportions. There was a greater amount of fixed acids in the glucose-free culture.

There was little difference in the amounts of gas produced in the two cultures at the end of 24 hours' incubation, but glucose increased the total amount formed by approximately 33%.

Tests for creatinin, amines and indol were made. Mono-amines were formed; the amounts of creatinin fluctuated slightly; and indol was absent from both cultures.

Under the conditions of the experiment the presence of glucose did not exert any effect on the production of toxin by B. botulinus. These results are in harmony with the suggestion that the toxin is an autolytic product.

HYDROGEN-ION STUDIES

X. THE PREPARATION OF A DRY POWDER WITH "AMBOCEPTOR" PROPERTIES

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Hirsch [1] describes the preparation of specific precipitin as a dry powder or solid, and it seemed probable that other immune substances could be prepared as solids in a similar way. This now has been done with the sheep cell hemolysin. Amboceptor rabbit plasma (citrated) is brought slowly to a reaction of P_H 6.8 by adding N/100 HNO_3. The liquid is allowed to stand in the icebox over night to permit as complete separation as possible of a fibrin-like clot, and then filtered. More N/100 HNO_3 is added to the clear filtrate until a maximum turbidity is obtained, and after standing in the icebox about an hour the finely flocculent precipitate which forms is filtered off. To the clear filtrate an equal volume of saturated ammonium sulphate is added. After complete globulin precipitation, the liquid is filtered through a soft filter, the white precipitate washed with a half saturated aqueous solution of ammonium sulphate, and then with a saturated aqueous solution of sodium chloride. The residue on the filter paper is dissolved in a volume of distilled water equal to twice that of the plasma used. To approximately each 10 c.c. of the aqueous extract is added 0.5 c c. N/10 HCl drop by drop and usually from 1 to 3 c c. of saturated sodium chloride solution. A white flocculent precipitate settles out rapidly. This is brought by centrifuging into the bottom of one tube; the supernatant liquid of the last portion added is poured off; the tube containing the centrifuged precipitate is inverted, and the small amount of liquid present is drained off. The protein precipitate in the tube is dried in a vacuum desiccator until it becomes of brittle, putty-like consistency, when it is removed, divided into small particles on a watch crystal, thoroughly dried, and stored in suitable glass containers in a desiccator. Some difficulty was experienced with the amboceptor and precipitin preparations in obtaining readily soluble products.

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[1] Jour. Infect. Dis., 1923, 33, p. 470.

A helpful suggestion by Professor Julius Stieglitz concerning the possible formation on the protein particles of a water film prompted a revision of the technic immediately preceding the drying. As a result, easily soluble preparations have been obtained according to the method here described. When dried as described the precipitate is a white, firm, brittle mass, slowly soluble in water like gelatin, forming a clear, slightly viscid liquid. The solubility of the dried substance is increased by adding to the distilled water used as solvent N/10 NaOH, drop by drop, enough so that when the substance is dissolved completely the liquid is neutral or faintly alkaline to litmus.

The reactions for one dried preparation are summarized as follows:

Seven mg. of a recently prepared solid amboceptor substance were dissolved in 2 c c. of distilled water and neutralized to litmus with 0.2 c c. N/10 NaOH. Quantities of a 1 : 10 dilution of this solution as small as 0.2 c c. with 2 units of guinea-pig complement were hemolytic in 30 minutes for 0.5 c c. of a 5% sheep cell suspension. No hemolysis occurred in control tubes containing respectively amboceptor solution and sheep cells, complement and sheep cells, and salt solution and sheep cells. The estimated quantity of dried substance present in the highest dilution causing complete hemolysis is 0.064 mg.

SUMMARY

Sheep cell hemolysin (amboceptor) may be prepared as a solid according to the method outlined.

The amboceptor so prepared is a protein-acid salt, soluble in water, forming a liquid with an acid reaction. This liquid made neutral to litmus with a small amount of N/10 NaOH is hemolytic in high dilutions for sheep cells in the presence of complement.

A NEW METHOD OF STAINING ACID-FAST BACTERIA AND SPORES

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A review of the numerous methods of staining the tubercle bacilli discloses that each of the steps in the staining has been subjected to numerous modifications. Which of the methods, if any, give results superior to the Ziehl-Neelson method remains to be determined. Certain of the modifications, however, while possibly not giving superior results, may have certain advantages as regards convenience, time and cheapness. The present paper describes the use of acetone and iodine acetone as the decolorizer in the differential staining of tubercle bacilli and of bacterial spores. Notes on other factors affecting the staining of tubercle bacilli are included.

METHOD OF STAINING TUBERCLE BACILLI

1. Place slide in a beaker of steaming carbol-fuchsin (1.5 gm. basic fuchsin, 15 c c. of 95% alcohol, 5 c c. carbolic acid crystals, 85 c c. distilled water) for 2 minutes or longer.

2. Wash without allowing smear to become dry.

3. Decolorize with acetone or iodine acetone (1 part Gram's iodine solution to 3 parts acetone, or add a few crystals of iodine to acetone) until the smear is nearly colorless. This requires only a few seconds if the smear has not been allowed to dry. If acetone without iodine is being used and does not decolorize the smear quickly and sufficiently, flood slide with Gram's iodine solution, immediately drain off and repeat acetone treatment. Flooding slide with the decolorizer twice, tipping it back and forth each time and washing in water is usually sufficient. Ignore color of thick lumps of material on smear. The slide can be dipped in water at any time to determine whether it is properly decolorized.

4. Counterstain with 10% aqueous solution of a saturated alcoholic solution of methylene blue for from 1 to 2 minutes or a saturated aqueous solution of picric acid for from 5 to 10 seconds.

The ideal primary stain is one that penetrates the acid-fast organisms rapidly, is stable in solution, is readily soluble in the decolorizer and does not become insoluble on exposure to heat. The carbol-fuchsin solution commonly used penetrates well. Most directions call for steaming from 5 to 10 minutes. Keilty[1] and later Hager and Dersch[2] state that steaming is not necessary, that exposure to cold carbol fuchsin for from 5 to 10 minutes is sufficient for penetration. It has been our experience in comparing smears stained in steaming

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[1] Jour. Am. Med. Assn., 1916, 66, p. 1619.
[2] Ibid., 1923, 80, p. 460.

and cold carbol-fuchsin that steaming is not necessary but that heat hastens the penetration of the dye. The time required for penetration can be reduced several minutes by steaming the carbol-fuchsin. Steaming from 2 to 5 minutes gives as good or better penetration than cold carbol-fuchsin in 15 minutes. We obtained good penetration in boiling carbol-fuchsin in from 10 to 60 seconds. What constitutes satisfactory penetration depends to some extent on the decolorizer used and its control.

Comparison of the keeping qualities of solutions made from 17 different samples of basic fuchsin demonstrated considerable variation, some solutions retaining their staining properties longer than others. Some of the samples contained much inert matter, a heavy percipitate forming in a few weeks, and the solution becoming colorless. The formation of a precipitate is not objectionable if the solution retains its color and staining properties. One of our samples, obtained from the Harmer Laboratory, was used, steaming, almost daily for a year. The water lost by evaporation was replaced. At the end of this time there appeared to be no loss in the staining qualities of the solution. The keeping qualities of the solutions from the 17 samples were compared under identical conditions, the Harmer and Grübler samples being more stable than the others. With more care in the preparation of the solutions, especially in the use of clean glassware and pure water, other samples of fuchsin may prove to be equally satisfactory. Pickett[3] records a solution of carbol-fuchsin, made from Grübler's basic fuchsin, retaining its staining properties for 19 years. It is his opinion that the stability of dye solution depends on the use of high grade chemicals, clean glassware and pure water. Alkali causes the solution to break down.

The solubility of the dye in the decolorizer is of considerable importance. A dye which forms little or no precipitate on the slide and rapidly goes into solution in the decolorizer is to be preferred. A dye that produces a dirty, sticky looking, heavily stained smear and goes into the decolorizer slowly is less likely to give satisfactory and clear cut results. Prolonged exposure to the decolorizer and counterstain is necessary when using such dyes. It is always advisable to modify the technic to meet the conditions presented by the dye used.

Many methods for the differential staining of tubercle bacilli warn against boiling the dye. There is no serious objection to staining with boiling carbol-fuchsin provided the smear is not permitted to dry. Heat hastens the penetration of the dye, but the smear is more likely to become dry if care is not observed. Drying affects the solubility of the dye in the decolorizer. If the drying occurs at room temperature no serious results follow as the dye will go into solution, though somewhat more slowly than if not dried. If the dye becomes dry at a high temperature, it becomes insoluble in the decolorizer, and the smear must be discarded. To avoid drying, the slide should be washed immediately after staining with the fuchsin. Boiling does not destroy the acid-fastness of the tubercle bacilli, does not make other organisms acid-fast and apparently does not affect the stability of the dye solution.

Our samples of fuchsin varied in their reaction to drying, especially at a high temperature, some becoming insoluble more rapidly than others. One sample remained soluble after drying at practically a boiling temperature. The slide was taken from boiling carbol-fuchsin and allowed to dry for several minutes over the flame of a Bunsen burner. Other samples of dye treated the same way became partially insoluble, and the smears had to be discarded. An examination of American basic fuchsins as regards penetration, stability under

[3] Trans. Am. Microsc. Soc., 1923, 42, p. 129.

favorable and unfavorable conditions, amount of precipitate under the conditions of staining, solubility in various decolorizers and reaction to heat should be made. A selection of the best dyes can be more satisfactorily made by this method than by comparing slides stained by some standardized technic.

An ideal decolorizer for the acid-fast stain should, take the dye into solution rapidly, and not decolorize the organisms. It should rapidly decolorize the mucous and nonacid fast organims. The solution should be stable. We believe that acetone or an iodine acetone solution more nearly meets these qualifications than any other described decolorizer. In testing acetone as a decolorizer in the differential staining of tubercle bacilli, we find that it has some advantages over acid alcohol and sodium sulphite. Acetone decolorizes the smear more rapidly than acid alcohol, and when iodine is utilized more rapidly than sodium sulphite, the resulting slide is usually cleaner, owing to a more complete washing out of the dye. This is especially true of slides that have become dry. If the slide accidentally dries at a moderate temperature, the iodine acetone will decolorize it more rapidly and completely than either of the other decolorizers. The sodium sulphite decolorizer must be made up fresh every few days. The acetone or iodine acetone solution keeps indefinitely. The one objection to acetone is that it evaporates rapidly unless kept in a well stoppered bottle. Since acetone or iodine acetone serves as a superior decolorizer in the Gram stain, its use as the decolorizer in the acid fast-stain eliminates the necessity of having a separate decolorizer for the latter stain.

The iodine added to the acetone, or to the smear before the acetone, saturates the affinities of the dye and the dye in this condition is more readily washed from the slide and nonacid-fast organisms. The effects of the iodine may be obtained in several ways. The smear may be exposed for a moment to Gram's iodine solution and decolorized with acetone. The same effect may be obtained by adding Gram's iodine solution or a crystal of iodine to the acetone until the latter is slightly colored. If the iodine acetone solution is to be used as a decolorizer in the Gram stain, crystals instead of Gram's solution should be added to the acetone as a small amount of water in the acetone affects the Gram reaction. The presence or absence of a small amount of water in the acetone is immaterial in the acid-fast stain. Water tends to slow down the decolorizing process but at the same time reduces the possibility of the nonacid-fast gram-positive organism retaining the carbol-fuchsin. The iodine is not added as in the Gram stain to cause a percipitate in the cells. There is no danger that the iodine will cause any nonacid-fast organism to retain the fuchsin. Carbol-fuchsin under the most favorable conditions does not give a good Gram reaction. The iodine solution is too weak and the exposure too short to give a Gram reaction with the best methyl violet. The best methyl or crystal violet does not give a Gram reaction when exposed to a mixture of iodine and acetone. The acetone or iodine acetone does not decolorize the tubercle bacilli in the time required to decolorize the smear.

Smears of tuberculous sputum stained by our method show as many acid-fast organisms as when stained by the Ziehl-Neelson method. We have made no comparisons with smears stained by the Schulte-Tigges method in which sodium sulphite is used as the decolorizer. Sodium sulphite promises to give better results than acid alcohol.

The essentials of a good counterstain are that it does not stain the acid-fast organisms, that it covers over small amounts of basic fuchsin left on the slide in mucus or nonacid-fast organisms, and that it gives satisfactory contrast with the fuchsin. The choice of a counterstain apparently lies between methylene

blue and picric acid. Neither stain penetrates the acid-fast organisms sufficiently to cover over the fuchsin present. The methylene blue gives greater contrast than picric acid with fuchsin. The methylene blue also obscures the basic fuchsin left in the mucus better than picric acid. Methylene blue solutions for counterstaining are made up in various ways. Some of these should be more satisfactory than others. Results will vary with different samples of dye. We prefer an intensely staining counterstain. If the acid-fast organisms are well stained with the fuchsin and decolorized with acetone, there appears to be no danger of the counter stain obscuring the diagnosis. Shoub[4] found that picric acid gives better results as a counterstain than saturated aqueous methylene blue with the Ziehl-Neelson method. Picric acid solutions give results more uniform than those produced from different samples of methylene blue.

A combined decolorizer and counterstain consisting of a solution of dye in acetone may be used but does not give as satisfactory results as when the solutions are used separately.

SPORE STAIN

The method described for the staining of the tubercle bacilli, slightly modified, serves as a superior spore stain. The same solutions are used, and the statements made concerning them hold true for the spore stain.

In ordinary bacteriologic work the staining of spores is unnecessary, their presence being determined by a study of the morphology and lack of penetration of ordinary dyes. For a special study of bacteria and their spores it is often desirable that the spores be differentially stained. For this purpose the method described here has proved more satisfactory than any other method known to us. This method does not require special solutions and is comparatively rapid.

METHOD

(1) Place slide in beaker of steaming carbol-fuchsin (same solution as used in acid-fast stain) for from 1 to 2 minutes.

(2) Wash, care being taken to prevent drying before slide is cool.

(3) Decolorize with from 2 to 3 changes of acetone, leaving each change of acetone on the slide about 2 seconds. Acetone is more satisfactory than acid alcohol or sodium sulphite.

(4) Wash and counterstain with Loeffler's methylene blue for two minutes or with any good counterstain, as preferred. Methylene blue is preferred to picric acid as giving better contrast.

As pointed out by Burke[5] this method serves to differentiate the living spores from spores killed by heat, only the margins of the former taking the dye and the latter staining to the center. The heat of fixation apparently does not kill all the spores as the majority of them treated in the usual manner stain in the ring form, i. e., only the margins are stained. Increasing the exposure to heat will cause all the spores to stain solidly. With this fact in mind, the fixation of the smear can be controlled to produce the desired result.

[4] Jour. Bacteriol., 1923, 8, p. 121.
[5] Jour. Infect. Dis., 1923, 32, p. 433.

SUMMARY

Acetone, or iodine and acetone, serves as a superior decolorizer in the acid-fast stain as in the Gram stain. The same decolorizer can be used for both methods of staining.

There is no serious objection to exposing smears to be stained for the acid-fast organisms or spores to boiling carbol-fuchsin. Heat hastens the penetration of the dye. Satisfactory results can be obtained by an exposure of the smear to cold carbol-fuchsin.

Drying of carbol-fuchsin affects the solubility of the dye in the decolorizer. Drying at a high temperature causes the dye to become insoluble. Drying at room temperature has no serious affect on the final results if the described method of staining tubercle bacilli is used.

American basic fuchsins vary in their reaction to heat. Dyes that are little affected by heat are to be preferred.

Solutions of American basic fuchsins vary in stability. Alkali causes the solutions to break down.

The method of staining spores here described makes it possible to distinguish readily living spores from spores killed by heat.

OCCURRENCE AND STAINING OF LEPTOSPIRA ICTEROIDES IN GUINEA-PIGS INOCULATED EXPERIMENTALLY, WITH A STUDY OF THE LESIONS PRODUCED

With 3 Plates

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In 1907, Stimson [1] found an organism by the Levaditi method in the kidney taken from a yellow fever case. He stained and examined the brain, liver, heart and kidney; nothing of special interest was found in the first three tissues, but in the kidney a definite organism was demonstrated. This organism he described as opaque black, in general appearance suggesting a spirochete, irregularly curved, one or both extremities often bent to form a hook. It appeared to be confined to the cells and lumina of the tubules of the kidney, none appearing in the blood vessels, glomeruli or interstitial tissue. For this organism he suggested the name Spirochaeta interrogans, because of its hooked appearance.

Noguchi,[2] in his work in the commission sent to Guayaquil, Ecuador, by the International Health Board of the Rockefeller Foundation, in 1918, when he found the supposed causative organism of yellow fever, stated that the morphologic features of Leptospira icteroides are almost indistinguishable from Leptospira icterohemorrhagica or Leptospira hebdomadis. He did not, however, mention anything in regard to the staining properties of the yellow fever organism. As to the lesions produced by it, icteroides is predominantly icteronephritic, and has a marked tendency to produce fatty degeneration of the parenchyma of the liver and kidney. Hemorrhage of the mucous membrane is more marked in experimental icteroides infections than in experimental infectious jaundice. In men, yellow fever is more rapid in its course and has a higher mortality rate than has infectious jaundice.

In Noguchi and Kligler's [3] report of their experimental studies of yellow fever in Merida, Yucatan, they state that the organism from the

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[1] Pub. Health Rept., U. S. Mar. Hosp. Serv., 1907, 22, p. 541.
[2] Lancet, 1922, 1, p. 1185.
[3] Jour. Exper. Med., 1920, 32, p. 601.

blood and from liver emulsions of yellow fever patients failed to stain with ordinary dyes. Further, the organisms often occurred in such small numbers in the blood of patients that they were missed in dark-field preparations. In guinea-pigs inoculated with blood and with liver emulsions from patients dying with yellow fever, small hemorrhagic and congested areas were found throughout the lungs; the liver was smaller in size than normal and appeared reddish brown with yellow mottlings; the kidneys were paler than normal. By the Levaditi method Leptospira icteroides was found to be fairly numerous in the liver and kidney.

Hoffman [4] reported a detailed study of the histopathology and hematology of guinea-pigs infected with Leptospira icteroides (Merida strain), examining, in all, 40 animals. The tissues were fixed in formol and stained with hematoxylin and eosin. He found the general structure of the liver slightly altered and quite unlike the liver of human yellow fever with its marked destruction of parenchymatous cells. In the guinea-pig liver there were few vacuoles and no zonal degenerations in the lobules. The cells showed cloudy swelling and numerous slight hemorrhages between the cells. Sudan staining showed only a slight fatty change. The kidney when stained with sudan showed large or small fat droplets, often covering the nucleus. The epithelium of the tubules showed cloudy swelling, and desquamated epithelium often obliterated the tubuli. There were numerous small hemorrhages in the stroma of the kidney, between the tubules. The spleen showed a slight infiltration of the follicles with a proliferation of the trabecular tissue. The blood sinuses were filled with many large polymorphonuclear leukocytes. The lymph nodes showed changes almost similar to those seen in the spleen. Scattered irregularly throughout the lungs were numerous hemorrhages with the red cells filling the alveoli or interalveolar septums. Occasional small submucous hemorrhages appeared in the bronchi and trachea. The heart muscle fibers were swollen and cloudy, and contained small vacuoles. Hemorrhages appeared between the fibers.

Hoffman carried out similar experiments on guinea-pigs infected with Leptospira icterohemorrhagica. In 40 or 50 cases he found no detail differing from the findings in the Leptospira icteroides animals. In conclusion, he states that the tissue changes in experimental icteroides

[4] Jour. Trop. Med., 1922, 25, p. 353.

infections are very different from those in yellow fever in man. The liver changes are slight as compared with the severe parenchymatous changes in the latter disease, with no liver cells left in some human cases. He stated that destruction of red blood cells in the spleen by phagocytosis is not found in human yellow fever. The severe hyaline degeneration of voluntary muscles in the icteroides guinea-pig has never been mentioned in the study of human yellow-fever tissue. The changes in the intestines resemble the medullary infiltration of typhoid fever and are not seen in human cases. The severe degeneration of the blood does not occur in human yellow fever. The occurrence of hemorrhage is not so pronounced in human yellow fever. Hoffman regards the changes in the tissues of experimental icteroides animals as typical of Weil's disease in man. He considered it quite remarkable that the organisms of two diseases so different in man produce identical clinical and anatomic effects in experimental animals.

Perrin [5] also studied the hepatic lesions of experimental yellow fever, from a strain of Leptospira icteroides isolated in Vera Cruz by Grovas. He found fatty degeneration affecting the entire protoplasm and nucleus of the hepatic cells and destroying extensive regions of glandular parenchyma. The hepatic lesions are both congestive and hemorrhagic. The tissue lesions did not differ in nature from those of animals succumbing to experimental infectious jaundice, but they did differ notably in intensity. He believes that neither in disposition, extent nor severity can the hepatic lesions of experimental yellow fever in the guinea-pig be differentiated from those of human yellow fever. In conclusion, he states that the chief diagnostic findings of the hepatic lesions of experimental yellow fever, besides the fibrous reactions of the stroma and vascular walls, are: (1) profuse fatty degeneration, which may affect the entire lobule, with central, intralobular or hepatic congestion predominating over peripheral, interlobular or portal; (2) insular necrosis.

Because of the few observations and the difference of opinion resulting therefrom, it was considered worth while to study the occurrence and distribution of another strain of Leptospira icteroides in the tissues of experimental animals, and the nature of the pathologic lesions in such animals. To the department of bacteriology (Professor Novy) we are indebted for our material, which was obtained from guinea-pigs

[5] Am. Jour. Trop. Med., 1923, 3, p. 27.

inoculated experimentally with a Peruvian strain of Leptospira icteroides. The transmissions were made from guinea-pig to guinea-pig every 5 days, with liver emulsions. We first stained the organisms in smears from liver, spleen, kidney and lymph glands by the Warthin-Starry [6] silver-agar method. Stains were made of emulsified liver also, but fewer organisms were found in these than in direct liver smears.

The method of staining smears is as follows:

1. Thoroughly air dry smears.
2. Fix in absolute alcohol for 3 minutes.
3. Wash in distilled water and dry.
4. Immerse coverslip in 2% silver nitrate and cover with a clean coverslip, also wet with silver nitrate so that they are held together by capillary attraction. Place upright against the side of a dark covered bottle containing not enough silver nitrate (2%) to cover.
5. Let stand in silver nitrate in a warm place for 2 hours, then remove the coverslips, separate and place the smears in the following reducing mixture:

2% silver nitrate solution	3 c c.
Warm glycerol	5 c c.
Warm 10% aqueous gelatine	5 c c.
Warm 1.5% agar solution	5 c c.
5% aqueous hydroquinone	0.8 to 1.00 c c.

6. Reduce until the smear as well as the reducing substance is a dark brown.
7. Rinse in 5% sodium thiosulphite solution.
8. Rinse in distilled water. Carefully wipe off any precipitate on the coverslip.
9. Air dry and mount in balsam.

Leptospira icteroides stains an intense black by this method, in sharp contrast to the gray or light brown background. The organisms show the typical shepherd's crook as well as straightened forms and circles.

The Warthin-Starry silver-agar method was found quite as successful for Leptospira icteroides in tissue as for Spirochaeta pallida. Small pieces of guinea-pig tissue were placed in 10% neutral formol immediately on removal from the animal. Fixation in formol was continued for 3 days to insure the best possible conditions for staining. The tissue was then dehydrated, infiltrated, embedded in paraffin and blocked in the usual manner. The blocks were cut 5 microns thick and the sections mounted on No. 1 coverslips, which had been spread with a minimum amount of albumin fixative. Unlike the method outlined for smears, the coverslip, after removal of the paraffin (by xylol and alcohol), is washed in distilled water and then placed in a concentrated solution of hydrogen peroxide for 10 minutes, to clear the background and make the organisms stand out more prominently. After again washing in distilled water

[6] Jour. Am. Med. Assn., 1921, 76, p. 234; Jour. Infect. Dis., 1922, 30, p. 592.

the coverslip is placed in 2% silver nitrate according to directions for the silver-agar stain. The Leptospira appears very black against a light brown or grayish background.

Altogether, tissues from 19 guinea-pigs inoculated with Leptospira icteroides were stained and examined. In each of the 19 cases Leptospira was found in large numbers in the liver. They were scattered between the liver cells everywhere, and not limited to particular areas. They also appeared in large numbers in and around the hepatic trinities. The fresh liver tissue was extremely friable. With a hematoxylin and eosin stain the liver showed marked acute congestion, and occasional small hyaline thrombi were scattered through the liver capillaries. There was an increased number of leukocytes in the capillaries. The liver cells showed marked cloudy swelling and dissociation with little fatty change. There was swelling of the stellate reticulo-endothelial Kupffer cells with phagocytosis of the leukocytes. There also was slight small cell infiltration of the periportal tissue.

In the lung the organisms were found in the bronchi, in the epithelium lining the bronchi, in the tissue surrounding the bronchi, and also in and around all hemorrhagic areas which may occur around the bronchi as well as scattered throughout the lung. In cases showing a large amount of hemorrhage, Leptospira appeared in large numbers, while in the others just a few organisms were found. In the hematoxylin and eosin stain the lung tissue showed marked acute congestion with numerous areas of hemorrhage, the hemorrhage in the majority of cases being localized in the neighborhood of medium sized bronchi. Many parts showed diffuse diapedesis and edema.

Leptospira organisms were demonstrated scattered in various parts of the spleen, in the areas of hemorrhage and often in groups in and directly around the trabeculae. In some cases the organisms were quite numerous in the spleen, but in none were they as numerous as in the liver, while in three cases they were quite scarce. The spleen showed marked acute passive congestion and increased hemolysis, with numerous hemophages containing red blood cells.

The inguinal lymph glands showed a few widely scattered organisms. In 6 of the 19 guinea-pigs no leptospiras were found in the lymph nodes, while in 6 other cases they appeared to be quite numerous. The lymph nodes with positive findings were greatly enlarged and congested and the loose tissue surrounding them markedly hemorrhagic. They showed marked acute congestion and marked sinus catarrh. There was also marked proliferation of the reticulo-endothelial cells, and great numbers of hemophages containing red blood cells in varying stages of disintegration.

In the pancreas, numerous leptospiras were demonstrated scattered throughout the connective tissue. The pancreas showed acute congestion with an increase in leukocytes in the blood vessels. There was edema of the interlobular tissue but no parenchymatous change.

In the heart muscle leptospiras were found widely scattered between the muscle cells, but never in large numbers. The heart muscle showed Zenker's necrosis most marked in the muscle trabeculae on the endocardial side. There was slight fatty degenerative infiltration under the endocardium and fairly marked in some of the papillary muscles. There was also a slight degree of leukocytosis in the capillaries.

A few leptospiras were found in the hemorrhagic areas in the subcutaneous fat. One of the guinea-pigs showed numerous marked subcutaneous hemorrhages. On staining these areas, several leptospiras were found grouped around the hair follicles and others widely scattered through the dermis.

Leptospira icteroides was also demonstrated to be quite numerous and widely scattered in the loose tissues of the eye and in hemorrhagic areas around the eye. The eye showed congestion but no other tissue change.

In the brain and spinal cord only a few leptospiras were demonstrated. The fibrillar network of the brain tissue takes the silver stain quite heavily so it is difficult to demonstrate the leptospiras, which of course are more or less straightened with curling ends, similar to the fibrils themselves. In an effort to make the background take up less silver and thereby allow the leptospiras to stand out more in contrast, the section was allowed to remain in hydrogen peroxide for a longer time than the other tissues, from 20 to 30 minutes. This was fairly successful, but only a few organisms could be demonstrated. The brain tissue showed acute congestion but no hemorrhage.

In the kidney the leptospiras were quite as numerous as in the liver, but were far more numerous than in the spleen or lung. The organisms appeared in the intertubular interstitial tissues and in the walls and lumina of the convoluted tubules. In the lumen of one tubule as many as 13 distinct leptospira were found. Only an occasional organism was found in the glomeruli, although a number were found directly around the glomeruli. Few leptospiras were found in the loops of Henle, in the straight tubules or in the medullary pyramids. Almost no fragmented forms were found in the kidney; even those in the lumina of the tubules were quite perfect and of characteristic form. The kidney showed acute passive congestion and cloudy swelling of the tubular epithelium. The most marked cloudy swelling was in the proximal portion of the convoluted tubules. There were numerous hyaline casts and occasional hemorrhages into the collecting tubules. There were occasional small hyaline thrombi. There was slight fatty change of the epithelium of the convoluted tubules. The subpelvic mucosa showed edema, congestion, hemorrhage and leukocyte infiltration.

Warthin,[7] in his report on his study of the excretion of Spirochaeta pallida through the kidney, says that he believes spirocheturia is a characteristic of the entire group of spirochetal infections. The finding of vast numbers of Leptospira icteroides in the kidney tubules would tend to indicate that it conforms to this characteristic in common with Spirochaeta pallida and Leptospira icterohemorrhagica.

CONCLUSION

It would appear from the preceding that Leptospira icteroides occurs in all tissues and especially wherever there are hemorrhagic areas. The organisms are found in greatest numbers in the liver, in large numbers in the kidney, to a less extent in the spleen and lung, while in other tissues they are more widely scattered.

We did not find the marked fatty degeneration of liver and kidney which Noguchi and Perrin report. The fatty change in both liver and

[7] Ibid., p. 569.

kidney was slight. There was acute congestion of all organs, most marked in the liver, kidney, spleen, lung and lymph nodes, and also cloudy swelling of the liver and kidney cells. The heart muscle showed Zenker's necrosis. Phagocytosis of the leukocytes was fairly marked, and there was slight leukocytosis.

The tissue changes found in these experimental yellow fever cases were not as marked or as severe as has been reported in human yellow fever cases, and do not differ in type of lesion from those of experimental infective icterus.

Leptospira icteroides stains as easily and as well as Spirochaeta pallida with the Warthin-Starry silver-agar stain.

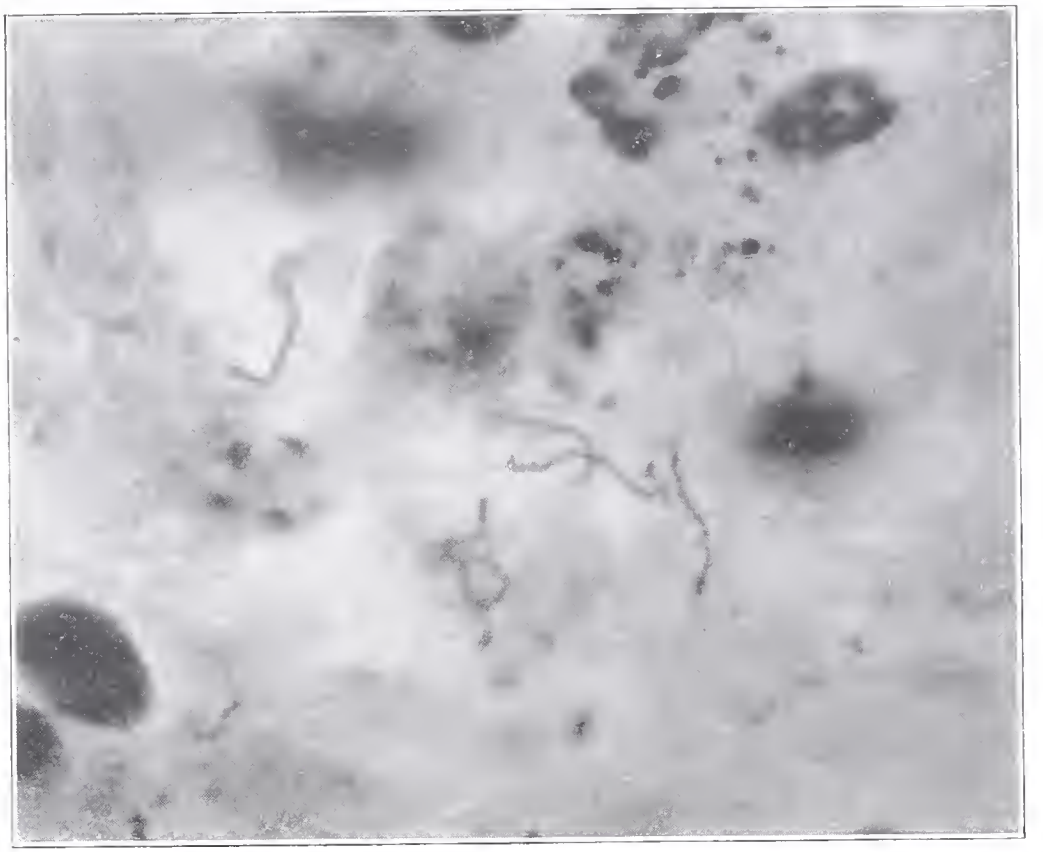

Fig. 1.—Leptospira icteroides in guinea-pig liver. Section stained by Warthin-Starry silver-agar method. Zeiss oil-immersion 2 mm., compensating ocular No. 4, bellows-length 155 cm.

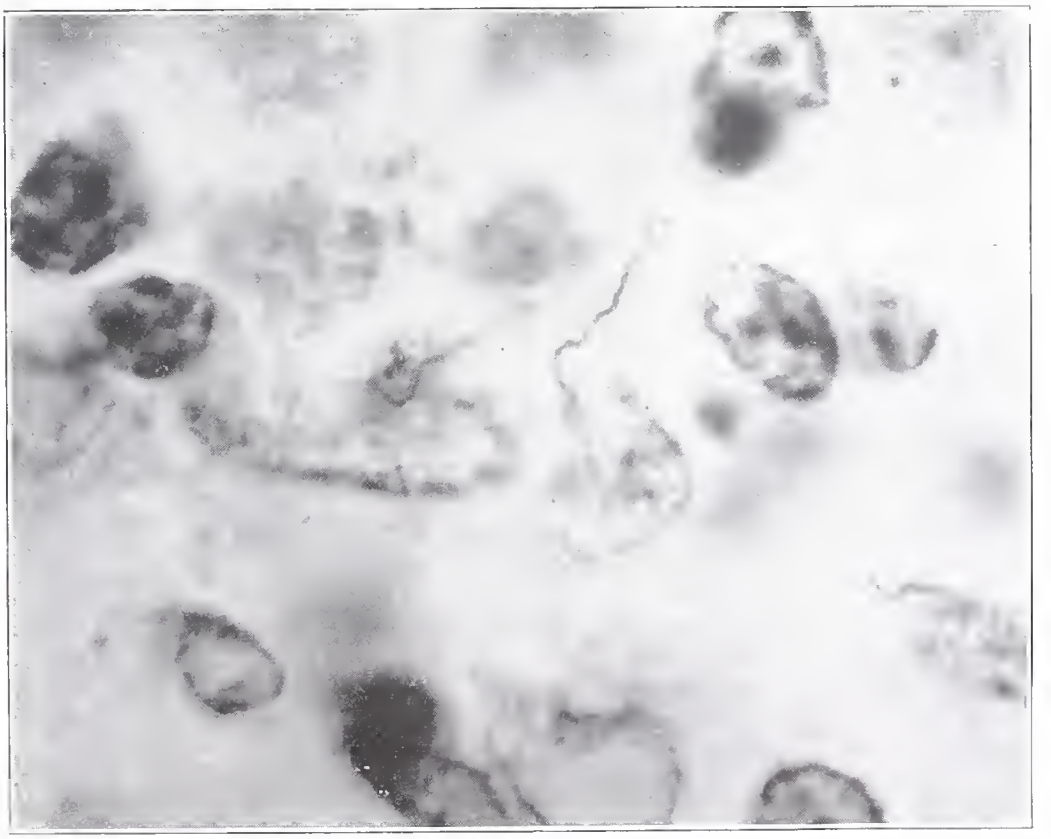

Fig. 2.—Leptospira icteroides in guinea-pig kidney. Section stained by Warthin-Starry silver-agar method. Zeiss oil-immersion 2 mm., compensating ocular No. 4, bellows-length 155 cm.

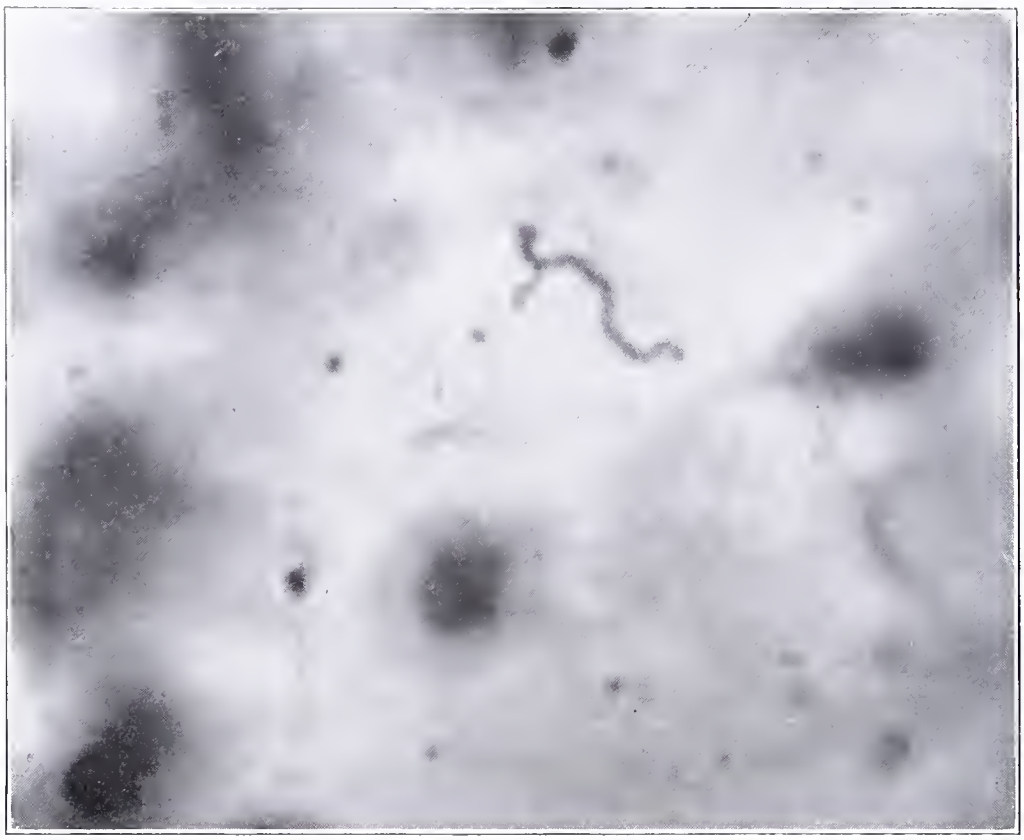

Fig. 3.—Leptospira icteroides in tubule of guinea-pig kidney. Section stained by Warthin-Starry silver-agar method. Zeiss oil-immersion 2 mm., compensating ocular X 10, bellows-length 155 cm.

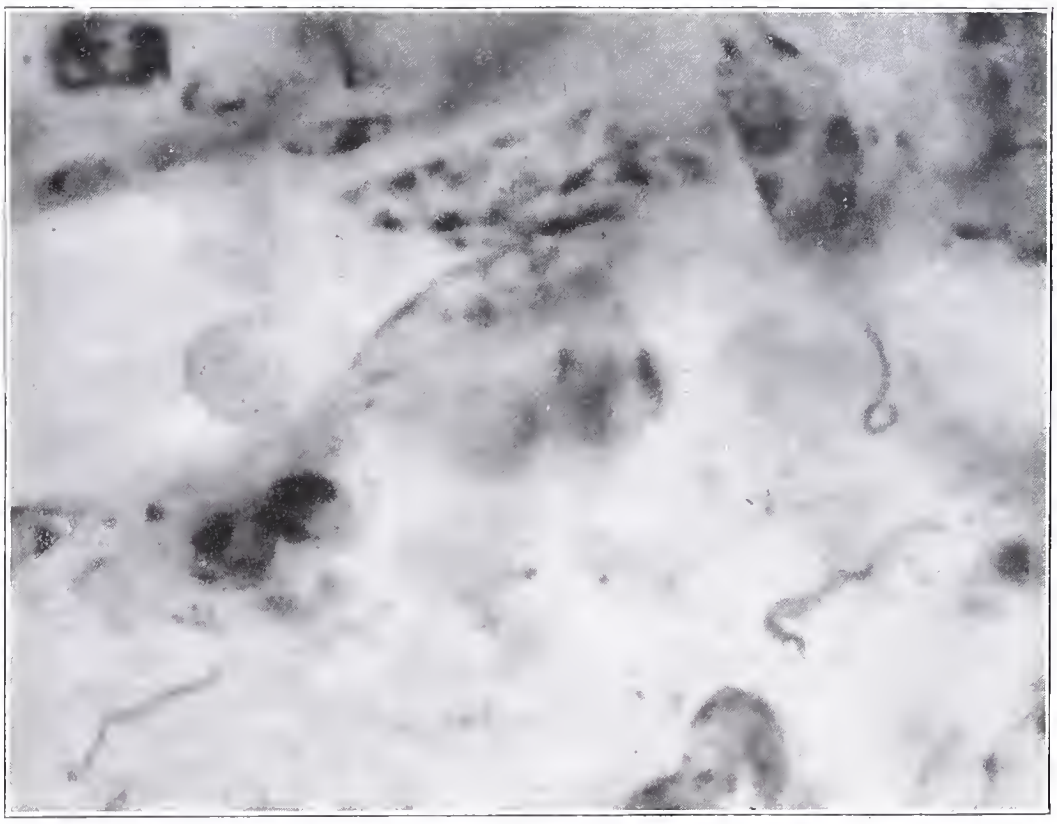

Fig. 4.—Leptospira icteroides in lung of guinea-pig. Section stained by Warthin-Starry silver-agar method. Zeiss 2 mm. oil-immersion, compensating ocular No. 4, bellows-length 155 cm.

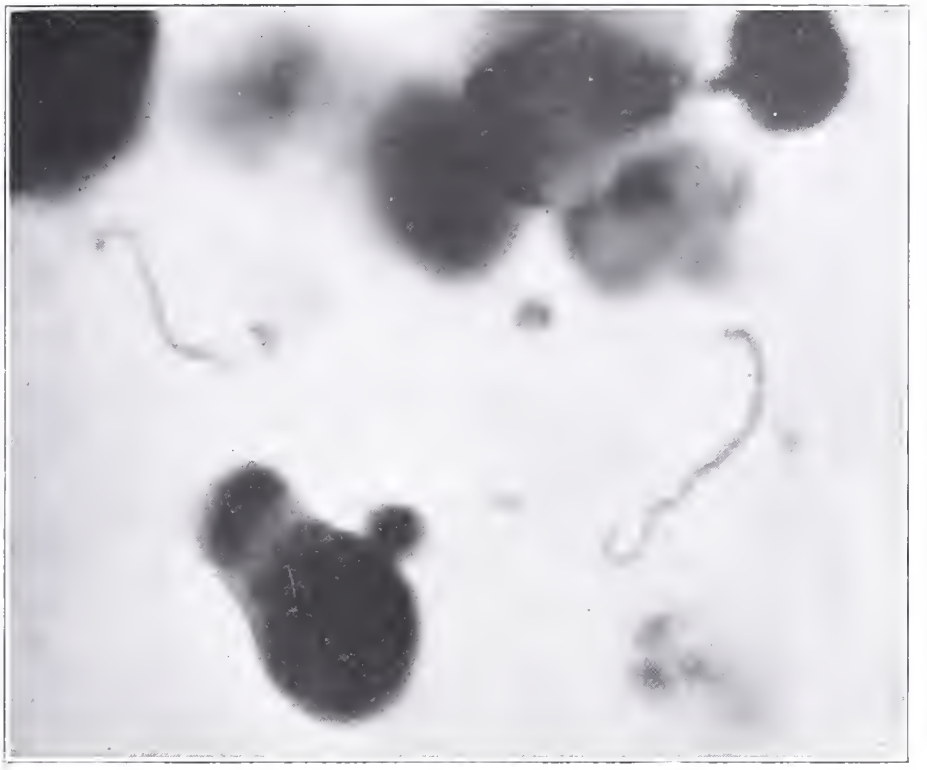

Fig. 5.—Leptospira icteroides in lymph node of guinea-pig. Section stained by Warthin-Starry silver-agar method. Zeiss 2 mm. oil-immersion, compensating ocular X 10, bellows-length 155 cm.

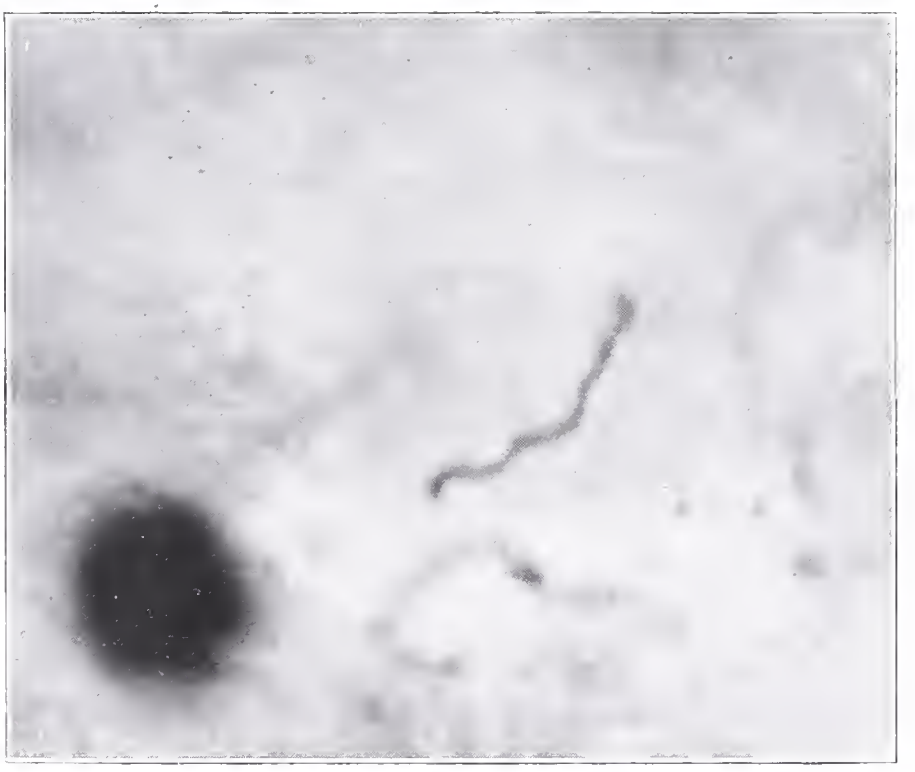

Fig. 6.—Leptospira icteroides in skin of guinea-pig. Section stained by Warthin-Starry silver-agar method. Zeiss 2 mm. oil-immersion, compensating ocular X 10, bellows-length 155 cm.

EFFECT OF BRIEF ANIMAL PASSAGE ON TWO STRAINS OF STREPTOCOCCUS

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Two distinct strains of streptococcus, one a typical Streptococcus hemolyticus, the other a typical Streptococcus viridans, have been kept under constant observation since Oct. 4, 1919. They were of peculiar interest in that they were isolated from the same vegetative growth on an aortic valve, which was obtained at necropsy from a typical case of acute and chronic infectious endocarditis. The 2 strains offered an unusual opportunity to study the influence of growth in the same host, in the same lesion, and in the same tissue on morphologic, bacteriologic, and serologic characteristics. The question also arose as to whether one strain might possibly be a variant of the other, since recent investigators have reported that hemolytic streptococci give rise to green-producing descendants and that green-producing streptococci split off hemolytic variants.

The two strains, as previously reported,[1] differed when first isolated and constantly thereafter in their virulence, in their morphology, in their reactions on blood and sugar mediums, and in their serologic reactions. At no time during a prolonged period of cultivation outside of the body or after an occasional animal passage for virulence tests did the hemolytic strain split off nonhemolytic fractions or did the green-producing strain give rise to hemolytic descendants. However, after reading the recent reports of R. Schnitzer and F. Munter,[2] it was decided to make an effort to obtain green-producing variants from the hemolytic strain and hemolytic fractions from the nonhemolytic strain.

Schnitzer and Munter concluded from their experience with a number of streptococcus strains that after every inoculation with hemolytic streptococcus of every grade of virulence, a lessened virulence of the injected organisms occurred during the first hours in the animal body, and that this lessened virulence could be demonstrated by a loss of hemolytic character.

Briefly, their technic was as follows: The transfer of the isolated strain under observation was alternated on goat-blood agar and horse-serum broth. They considered the lethal dose the amount of a 24-hour serum broth culture

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[1] Jour. Infect. Dis., 1922, 30, p. 289.

[2] Ztschr. f. Hyg. u. Infektionskrankh., 1921, 93, p. 96; 94, p. 107.

that would kill a mouse in 48 hours, and the dose they injected into the mice used in the experiments was smaller than this lethal dose. Series of mice were injected intraperitoneally under aseptic conditions with the chosen dose, and one mouse per hour was killed and examined. Cultures were made from the heart blood, from the peritoneal fluid, and from the ground-up kidney tissue. In cultures from the mouse killed in the first hour, hemolytic and green-producing streptococcus colonies could both be isolated as stated, and the investigators were able to keep each variety of culture constant, even after prolonged cultivation on artificial medium. The green variants were uniformly much less virulent than the parent hemolytic organism or the hemolytic descendants obtained from the same inoculation. They found that hemolytic streptococcus strains gave rise to nonhemolytic strains more readily than viridans split off hemolytic fractions, although after large intraperitoneal doses of green-producing streptococci, hemolytic variants could be split off after a brief sojourn in the mouse.

This technic was closely adhered to in the following experiment: The mediums used for the cultivation of the two streptococcus strains were human blood agar and ascites dextrose broth. Control plates of the blood and of the streptococcus strain used were made with each series of tests. In May, 1922, when the first attempts were made to change the hemolytic quality of the hemolytic streptococcus strain, the organism had little virulence for mice, since it required 0.5 c c. of an 18-hour ascites dextrose broth culture to kill a mouse in 48 hours. In the first series of tests 3 mice were injected with this dose. The first mouse was killed at the end of 1 hour, the second at the end of 5 hours, and the third at the end of 24 hours. The thorax of each mouse was opened under sterile conditions, and the heart blood was cultivated on blood agar (block plates) and in ascites dextrose broth. The peritoneal fluid was cultivated in similar mediums. At this time the kidney tissue was not cultivated. From the mouse killed after 1 hour, methemoglobin-forming colonies were obtained from both the heart blood and from the peritoneal fluid, but no hemolytic or green-producing streptococci appeared; from the second mouse hemolytic colonies only appeared; and from the third mouse hemolytic colonies and methemoglobin-producing colonies were obtained from the peritoneal fluid, green-producing colonies were obtained from the peritoneal fluid, and green-producing and methemoglobin-producing colonies were isolated from the heart blood. Colonies of the 3 varieties were picked and transferred from the blood-agar plates into ascites dextrose broth, and, after 18 hours, transfers were made from each broth culture on blood-agar plates. On these plates hemolytic colonies only were obtained. In the second series of tests, 5 mice were used, and the same dosage was employed. One mouse was killed per hour; hemolytic colonies and nonhemolytic colonies were obtained from the first 3 mice, but as in the previous tests, after the transfer into broth, and back to blood-agar, hemolytic colonies only appeared. In the third series of tests, 5 mice were injected with 0.25 c c. of an 18-hour broth culture, and they were killed at 1-hour intervals. Cultures from the mouse killed at the end of 2 hours, in this series, had 6 nonhemolytic streptococcus colonies, in addition to the hemolytic colonies. From all the other cultures hemolytic colonies only were obtained, and as in the case of the other nonhemolytic colonies isolated, the hemolytic quality quickly returned. A fourth series of 5 mice were injected with 0.25 c c. of this hemolytic streptococcus ascites dextrose broth culture, and on this occasion only hemolytic colonies were obtained from all cultures. In the fifth series of 5 mice, the dose of hemolytic streptococcus was increased to 1 c c.

of a similar ascites dextrose broth culture. Cultures from the heart blood and from the peritoneal fluid in each instance yielded hemolytic colonies only.

It was then decided to modify the test by using an attenuated culture. One mouse, therefore, was injected with 1 c c. of an 18-hour ascites dextrose broth culture, which had been heated for 10 minutes; a second mouse was injected with 1 c c. of a 10-day culture of hemolytic streptococcus; and a third mouse was inoculated with 1 c c. of a 10-day broth culture of hemolytic streptococcus, which had been centrifuged and washed and resuspended in salt solution. The mice receiving the two latter injections were very ill, and they were killed at the end of 3 hours, when they were apparently at the point of death. Controls of each streptococcus suspension, and cultures of the heart blood and peritoneal fluids of the 3 mice yielded hemolytic streptococcus colonies only. It was next attempted to increase the resistance of the mice to the strain of hemolytic streptococcus. On July 15, 1922, 4 mice were injected subcutaneously with 0.5 c c. of a homologous hemolytic streptococcus vaccine, (500,000 per c c.) and 2 mice were injected intraperitoneally with similar doses. One of the latter died in 24 hours; the other 5 mice survived and were apparently in good health at the end of one week. On July 22, 2 of the subcutaneously vaccinated mice and the 1 surviving intraperitoneally vaccinated mouse were injected with 1 c c. of an 18-hour ascites dextrose broth culture of the hemolytic streptococcus strain. One subcutaneously vaccinated mouse was killed after 1 hour, and the other 2 at the end of 2 hours. Cultures were made from the heart blood and from the peritoneal fluid of each of the 3 mice. Hemolytic streptococcus colonies only appeared on the blood-agar plates. The 2 remaining mice received a second subcutaneous vaccination on Aug. 1, with the same dose that was previously used. On Aug. 10, each received 0.5 c c. of a streptococcus culture, intraperitoneally. The mice were very ill, and they were killed in an hour and a half after injection, at which time they were scarcely breathing. Cultures from the heart blood and from the peritoneal fluid again gave rise to hemolytic streptococci only. In 32 mice used in a series of tests and in 3 mice used for the preliminary virulence tests, nonhemolytic streptococcus colonies were obtained in 6 instances. When the colonies were transplanted on blood agar, the hemolytic characteristic returned on the first or on the second subculture.

Most of the strains of streptococcus used by Schnitzer and Munter had been freshly isolated, and therefore it seemed possible that the failure to produce nonhemolytic variants from the hemolytic streptococcus strain used in the foregoing experiments might be due to the fact that the strain had been isolated several years and that it had been cultivated during that time on artificial medium. As a control to that factor. a series of 5 mice were injected with 0.05 c c. of an ascites dextrose broth culture of a freshly isolated strain of a virulent hemolytic streptococcus obtained in pure culture from an infected sinus. No nonhemolytic fractions were obtained from the necropsy cultures of this set of mice.

Th modifying effect of brief animal passage was next tried with the strain of Streptococcus viridans which had been isolated at the same time the hemolytic strain was obtained. Two sets of tests were made. Three mice were used in the first series, and 4 mice in the second series. The mice were injected intraperitoneally with a large dose (1 c c.) of an 18-hour ascites dextrose broth culture. They were killed 1 per hour, and cultures from the heart blood and from the peritoneal fluid in each case showed only the green-producing streptococcus.

Films from the peritoneal fluids were made in all the mouse necropsies. In films obtained from mice killed after 1 or 2 hours, there were a large number of large phagocytes, a few lymphocytes, and a few polymorphonuclear leukocytes. The streptococci in these films were quite numerous and appeared in chains and in diplococcus formation. Considerable phagocytosis was present. In the films taken from the mice killed after 4 to 5 hours, there was a predominance of polymorphonuclear leukocytes and fewer bacteria present. The bacteria in all the films varied in morphology, and many of them appeared swollen. There was also nonuniformity in staining; gram-positive and gram-negative cocci frequently appeared in the same field, and even at times within the same cell.

The experiments with the hemolytic streptococcus strain were repeated, using white rats as the host. Fifteen young rats were injected intraperitoneally with the 18-hour hemolytic streptococcus ascites dextrose broth culture. Five rats received a dose of 0.5 c c. each, 5 received 2 c c. of the original culture, and 5 received a dose of 1 c c. of a heavy sediment from the 18-hour centrifugalized streptococcus culture. A rat from each series was killed hourly, and peritoneal and heart blood cultures were made. In only one instance was there a nonhemolytic streptococcus isolated; that was in the heart and peritoneal cultures from the rat killed in 1 hour after the injection of 1 c c. of the heavy suspension of streptococcus sediment. The hemolytic quality of these colonies returned in 48 hours.

Nonhemolytic fractions could be obtained by the usual methods[3] of cultivating the hemolytic strain on salt-free agar to which blood had been added or on dextrose blood-agar or by streaking on blood-agar that had been heated to 80 C. The first subcultures of the nonhemolytic colonies produced hemolytic colonies on blood-agar plates.

TABLE 1

Results of Cultures

Hours after Inoculation	Streptococcus viridans Colonies	Streptococcus hemolyticus Colonies
1	6	2
2	5	0
3	0	3
4	0	1
5	3	0

When a few nonhemolytic colonies were obtained in the first 2 series of mice injected with the hemolytic streptococcus strain and the transient nature of the mutation was not recognized, the question arose as to what the modifying factor might be. It seemed probable that fresh serum might cause the mutation; accordingly a mouse was bled from the heart under sterile conditions and the serum collected and heavily inoculated with the bacterial sediment from a centrifugalized 18-hour ascites dextrose broth culture of the hemolytic streptococcus strain. The inoculated serum was incubated at 37 C. and hourly for 5 hours films were made and cultures on blood-agar plates. The bacteria in the films showed swollen coccus forms, which stained both gram-positively and gram-negatively as in the case of the peritoneal films. The results of the cultures appear in table 1.

[3] Monograph of Rockefeller Institute for Medical Research, 1919, No. 9; Clawson, B. J.: Jour. Infect. Dis., 1920, 27, p. 368.

The control blood-agar plates made of the hemolytic streptococcus culture used had Streptococcus hemolyticus only. The uninoculated serum from the mouse was not cultivated, so that there was the possibility that the apparently normal mouse had a Streptococcus viridans bacteriemia. However, the green-producing colonies isolated reacted immunologically in the same way as the hemolytic streptococcus strain, a finding that corresponded with the observations of Valentine and Krumwiede,[4] who had a hemolytic strain that spontaneously on transfer developed a nonhemolytic fraction, the agglutination of which strain was identical with the hemolytic fraction. The results of the agglutination test of the green-producing streptococcus obtained from the mouse serum with the hemolytic strain and with the nonhemolytic strain antiserums, are shown in table 2.

TABLE 2

RESULTS OF AGGLUTINATION TEST

Antiserum	Bacterial Suspension	Agglutination
I	I	1-8000
I	III	1-1280
I	II	0
II	II	1-40
II	I	0
II	III	0

I, Streptococcus hemolyticus.
II, Streptococcus viridans.
III, Streptococcus hemolyticus (nonhemolytic variant).

Absorption tests with the green-producing strain were similar to those with the hemolytic strain. The green-producing strain maintained that characteristic for the 3 months in which it was under observation; at the end of that time the strain was lost.

Other serum tests were made using mouse serum, rabbit serum, human serum, and rat serum; but only hemolytic streptococcus colonies were obtained, although many modifications were tried. For example, one of the rat tests was as follows: 0.4 c c. of rat serum was inoculated with 1 loop of hemolytic streptococcus sediment; another 0.4 c c. of the same serum was inoculated with 6 loops of the hemolytic streptococcus sediment. Each inoculated serum was incubated at 37 C. and plated hourly for 5 hours. Hemolytic streptococci only were obtained on all plates. In another test, fresh sterile rat serum that was inoculated with 1 loop of hemolytic streptococci which had been heated for 15 minutes on culture gave hemolytic streptococci. Sterile fresh rat serum inoculated with hemolytic streptococci which had been treated with immune hemolytic streptococcus antiserum for 15 minutes gave no growth when it was plated on blood-agar.

The experiments were discontinued in August, 1922, because the two strains of streptococci under observation had not yielded constant variants with any of the dosages, hosts, or any of the other modifications tried. The freshly isolated hemolytic streptococcus also had not produced nonhemolytic fractions. However, after reading the third communication of Schnitzer and Munter,[5] in which they again affirmed that weakly virulent, partially virulent, and highly virulent hemolytic streptococci produced nonhemolytic descendants during the first 3 hours' sojourn in the mouse's peritoneum, it was decided to repeat the test with the hemolytic streptococcus strain used before. The kidney tissue,

[4] Jour. Exper. Med., 1922, 36, p. 157.
[5] Ztschr. f. Hyg. u. Infektionskrankh., 1923, 99, p. 366.

particularly in the tests of Schnitzer and Munter, seemed to give rise to nonhemolytic fractions; therefore in the experiments of July, 1923, the ground kidney tissue, as well as the heart blood and the peritoneal fluid, was cultivated in each mouse necropsy. The virulence of the culture at this time was as follows: 1 c c. (5,000,000 bacteria per c c.) of an 18-hour ascites dextrose broth streptococcus culture killed a mouse in 18 hours; 0.75 c c., in 24 hours; 0.5 c c., in 48 hours; and 0.025 c c. did not kill a mouse in 4 days. A small dose was used in the tests: 3 mice were injected intraperitoneally with ⅕ of the amount required to kill a mouse in 48 hours; 3 mice with 1/10 of that lethal dose: and 3 mice with 1/20 of the dose. Blood-agar plates of the kidney tissue, peritoneal fluid, and heart blood were examined at the end of 24 hours and again in 48 hours; hemolytic streptococci colonies only appeared on the blood-agar plates. The films from the peritoneal fluid resembled those previously described.

RESULTS

Of the 50 to 60 mice injected intraperitoneally with the strain of hemolytic streptococcus under observation, only 6 yielded other than hemolytic streptococcus colonies. From the 6 mice methemoglobin colonies or green-producing colonies were obtained. In every instance, after transfer into the ascites dextrose broth and replating the 18-hour broth culture on blood-agar plates, hemolytic streptococci only were obtained.

A freshly isolated virulent streptococcus injected intraperitoneally in 5 mice for brief periods produced hemolytic streptococcus colonies only.

The Streptococcus viridans strain under observation did not split off hemolytic fractions after brief periods in the peritoneum of each of 7 mice.

Films from the peritoneal fluid indicated that the morphology and staining properties of the streptococci were modified by the sojourn in the mouse. In early hours after injection, there were many streptococci and there was rapid phagocytosis, and in the later hours there were few bacteria and many polymorphonuclear leukocytes.

Of the 15 white rats injected with hemolytic streptococci, 1 only produced a few nonhemolytic streptococcus colonies. This variant recovered its hemolytic character in 48 hours.

Only one of the serums inoculated with the hemolytic streptococcus strain yielded a green-producing streptococcus. This retained that characteristic for 3 months. The uninoculated serum in that case had not been cultivated, so that it could not be proved that the mouse did not have a Streptococcus viridans infection, althuogh the results of the serologic tests were similar to those of the hemolytic strain.

The passage of the streptococci into the blood stream and tissue of the injected mouse was rapid, since cultures from the heart blood and from the kidney tissue made one hour after inoculation, in most cases, yielded streptococci.

CONCLUSIONS

Streptococcus hemolyticus isolated from the aortic heart valve did not produce variants of any stability after a brief sojourn in the peritoneum of a mouse. Streptococcus viridans isolated from the same heart valve split off no variants, nor did a freshly isolated virulent hemolytic streptococcus yield nonhemolytic descendants.

ACTION OF AMMONIA ON PNEUMOCOCCUS AND MECHANISM OF CAPSULE STAINING

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ACTION OF AMMONIA ON PNEUMOCOCCUS

During experiments on growing the pneumococcus in tandem with other bacteria, it was noted that it would not grow with a spore-forming ammonia producer. Experiments showed that a 1:1,000 or 1:1,500 dilution of ammonium hydroxide inhibited the growth in ascites dextrose broth. Since it was known that ammonia is excreted in the sputum after the administration of ammonium salts, I was inclined to believe that in addition to the expectorant action the NH_4 ion might

TABLE 1

Result of Experiments on Growing Pneumococcus with Other Bacteria

Dilutions	NH_4OH		NaOH		NH_4Cl	$NH_4C_2H_3O_2$	$NH_4C_3H_5O_3$	NH_4NO_3
	8 Hours	20 Hours	8 Hours	20 Hours	8 Hours	8 Hours	8 Hours	8 Hours
1 : 4	0	0	0	0	+	+	+	+
1 : 8	0	0	0	0	+	+	+	+
1 : 10	0	0	0	0	+	+	+	+
1 : 20	0	0 +	0	0 +	+	+	+	+
1 : 25	0	+	0	+	+	+	+	+
1 : 30	0	+	0	+	+	+	+	+
1 : 35	0	+	0	+	+	+	+	+
Control	+	+	+	+	+	+	+	+

exert an inhibitory action on the pneumococcus and thus further account for the popular and apparently beneficial action of these salts in colds and bronchitis. To test this more careful experiments were made. The following is representative:

N/2 solutions of ammonium hydroxide, chloride, acetate, lactate and nitrate were prepared. The salts of ammonia were made up by taking the same amount of NH_4OH and adding equivalent amounts of the acids and making up to a proper volume to make the solutions exactly N/2.

One c c. of each of these contained 0.0085 gm. NH_3. Their action along with that of N/2 NOOH was tested by adding 1 c c. of the

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various dilutions to 2 c c. of ascites dextrose broth inoculated with type 1 pneumococci. The cultures were incubated at 37 C.

Neutralizing the hydroxides with equal dilutions of HCl permitted growth throughout. Evidently the inhibitory action is due to the OH ion.

As shown by Coleman,[1] as much as 0.095 mg. of ammonia is excreted in 1 c c. of bronchial sputum when 5 grains of ammonium chloride is taken by mouth at 2 hour intervals (total 60 grains). Unless twice as much ammonia as reported by Coleman, who was merely interested in its expectorant action, can be excreted in the sputum, and

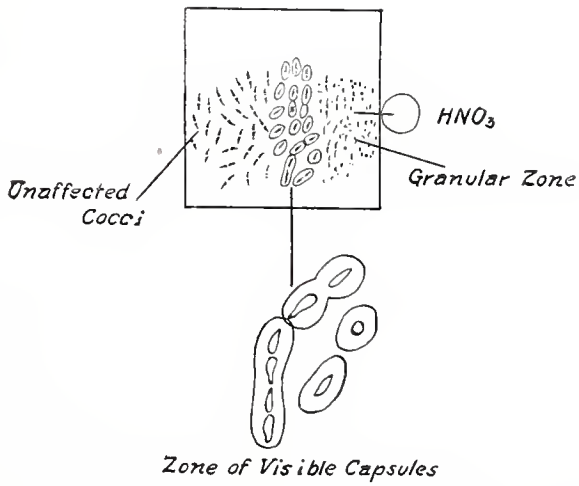

Fig. 1.—Showing the effect of acid on the capsules of pneumococci in a peritoneal exudate.

unless this ammonia is present entirely as the hydroxide, which is not at all certain, it cannot produce inhibition. According to the experiments, 0.28 mg. of ammonia as the hydroxide must be present to inhibit the growth of the pneumococcus.

Since ammonia is a constant product of autolysis,[2] it would be interesting to know whether it is present as the hydroxide and in sufficient amounts to inhibit the bacteria in resolving pneumonic exudates.

MECHANISM OF CAPSULE STAINING

The action of various acids on pneumococci in peritoneal exudates was tested as follows (fig. 1).

[1] Am. Jour. Med. Sc., 1916, 152, p. 569.
[2] Wells, H. G.: Chemical Pathology, 1920, p. 533.

If a drop of acid—pure hydrochloric, nitric, lactic, acetic—is allowed to run under an edge of the slip covering a microscopic preparation of peritoneal exudate, one can see the cocci near the acid disintegrate into fine granules. However, as the acid diffuses, and reaches the proper concentration, the hitherto invisible capsules leap into view in a most remarkable manner. As the concentration rises, these visible encapsulated forms are in turn dissolved, while new ones come into view. Experiments showed that the most favorable concentration for pro-

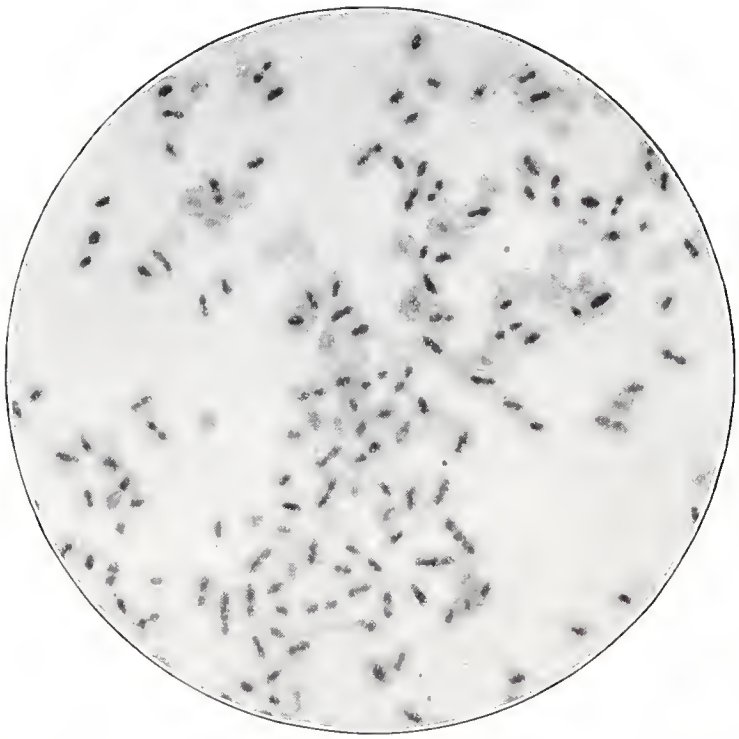

Fig. 2.—Washed cocci from peritoneal exudate mixed with equal parts of ascites fluid and N/20 HCl as in Rosenow's method, stained quickly with carbol gentian violet.

ducing this phenomenon is N/40 acid. If a loopful of peritoneal exudate is mixed with a loopful of N/20 HCl, all the capsules are visible. When such a mixture is spread in a thin layer, dried and fixed by heat, and stained quickly with carbol gentian violet, the cocci stain deeply while the capsular substance is stained a light purple (fig. 2).

Further experiments showed that the bacteria must be really encapsulated bacteria and that serum must be present before this reaction occurs. These experiments confirm the findings of Rosenow[3] and

[3] Jour. Infect. Dis., 1911, 9, p. 1.

further emphasize the colloidal nature of the staining process. This does not appear to be due to a mere change in optical property due to acid hydration of the capsular substance, but it appears as though at the right concentration of acid a change in the electric charge brings about a deposit of some constituent of serum upon the capsular substance, making it visible and weakly stainable by a basic dye and strongly stainable by an acid dye.

The following observations are of interest in this connection: (a) If a thin film of blood from pneumococcus septicemia is immediately placed in a moist chamber filled with osmic acid vapor for a minute or so, thoroughly washed with absolute alcohol and water and then stained with Giemsa's solution, the cocci stain a deep purple and the capsules a bright red. (b) if a rabbit, inoculated intravenously with a small dose of virulent pneumococci, receives an intravenous dose of 30 c c. of a 0.25% saturated aqueous solution of picric acid, in N/8 NaCl, it dies much sooner than controls of an intense septicemia, and in blood smears the capsules can be demonstrated easily with carbol gentian violet without the further addition of acid. Control animals only at times develop sufficient acidosis to enable one to stain the capsules without the further addition of acid, although this is possible when the animal has been dead for some hours; (c) when the pneumucoccus, pneumococcus mucous or B. pneumoniae are isolated on sugar-free medium, the capsules cannot be stained without the use of acid, but if sufficient acid is produced during growth it is not necessary to add more acid, although the further addition of serum may be necessary.

SUMMARY

Ammonium hydroxide inhibits the growth of the pneumococcus when as little as is represented by 0.28 mg. NH_3 is present in 3 c c. of ascites dextrose broth. Less than this quantity of NH_3 has been reported as being excreted in sputum after the administration of ammonium chloride to man by mouth. The inhibition is due to the OH ion.

When acids are allowed to diffuse under a coverglass preparation of a pneumococcus peritoneal exudate the capsules of the pneumococci become visible in a certain diffusion zone. Experiments showed that the optimum dilution of HCl was N/40. This phenomenon only occurs in the presence of serum or ascites fluid. If a mixture of equal parts of

pneumococci in diluted serum and N/20 HCl is spread in a thin film, fixed by heat, and stained quickly with carbol gentian violet, the capsules are stained. This is E. C. Rosenow's method. It is suggested that the capsules become visible and stainable because at a certain acid concentration there is a precipitation of some constituent of serum upon the capsular substance.

GROWTH OF CLOSTRIDIUM BOTULINUM IN FERMENTED VEGETABLES

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The many recent outbreaks of poisoning due to the toxin of Clostridium botulinum with the large percentage of fatal cases demand a careful survey of every state to determine with certainty the distribution of the organism, the variation in types with their toxins, and the conditions under which these types grow and produce their toxin. The cases of botulism in man which have been described in United States have been almost wholly the result of consumption of spoiled canned goods. Canned goods of certain kinds often offer ideal conditions for the growth of the organism on account of the absence of air, but while the organism is usually described as an anaerobe, it does grow in suitable mediums in varying amounts of oxygen. Among animals, silage has not infrequently been found to contain the toxin of this anaerobe. It has generally been accepted that it requires a medium nearly neutral in reaction for growth, but again this does not seem to be an absolute requirement since two deaths have resulted from the consumption of tomato-onion chili sauce,[1] a food product which is never neutral in reaction but always more or less acid. The fact that it produces its toxin in silage which is acid may be considered at first as another example of growth in an acid medium. In this case, however, it is possible that the toxin is produced before any considerable acidity is attained in the fermenting material. Bronfenbrenner and Schlesinger [2] have shown that the potency of the toxin is increased many times by even slight acidification, and hence it is possible that if a very small amount of toxin was formed in the silage the acid which results from the growth of other organisms might cause the material to become very toxic.

Some consideration of these facts, namely, growth of Clostridium botulinum in an acid medium and in a material not sealed from the air, suggested that there might be growth and toxin production in vegetables fermented for human consumption. Sauerkraut is often eaten raw, hence it seemed there might be a considerable element of

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[1] Cutter, J. B.: Jour. Am. Med. Assn., 1922, 79, p. 825.
[2] Proc. Soc. Exper. Biol. & Med., 1921, 19, p. 1.

danger. Green and yellow beans are often preserved by fermentation like that of sauerkraut, and these are sometimes eaten raw.

To determine whether Clostridium botulinum could grow and produce its toxin in a fermented food, fresh cabbage and green beans were used. The cabbage was cut into small pieces by passing it through a meat grinder. This also expressed some of the juice which was added to the chopped cabbage. The beans were cut into thin pieces. To both beans and cabbage salt was added in the amount of 2.5% by weight. Through the action of the salt and a little pressure a considerable amount of juice was extracted. Ten c c. of bean and of cabbage juice was placed respectively in each of 4 large test tubes, capacity 150 c c. Tube 1 of each set received an inoculum of 1 loop of material from a meat culture of Clostridium botulinum; tube 2 of each set received one loop from tube 1, and tube 3 received 3 loops from tube 2. The 3 tubes differed in the heaviness of inoculation and in initial toxin content, tube 1 receiving the largest amount and tubes 2 and 3 succesively less. Tube 4 of each set served as controls. The tubes of each set were then filled respectively with cabbage and with beans. The material was packed in tightly to exclude the air. A porcelain weight kept the vegetables approximately 2 inches below the surface of the liquid. The tubes were covered with tinfoil to keep out dust and to prevent evaporation to some extent. In order to prove the growth of the organism and determine whether toxin was produced, it was necessary to test the tubes at once for any initial toxicity. The manner in which the inoculation had been made and the subsequent packing into the tube of the cut vegetables should have insured a fairly even distribution of organisms and any toxin which was carried over in the inoculum. Guinea-pigs were forcibly fed with 2 to 3 c c. of the liquid from each tube, care having been taken to obtain the sample of liquid with a sterile pipet from near the bottom of the tube. In no case did any of the guinea-pigs exhibit any indication of illness following the feeding. The tubes of vegetables were left at room temperature and allowed to ferment. Fermentation followed rapidly. After a period of 2 months the material was well fermented. At this time guinea-pigs were again forcibly fed with 2 to 3 c c. of the liquid obtained as before from near the bottom of the tubes. The guinea-pigs which were fed the liquid from the fermented cabbage remained well. The results with 4 different lots of cabbage prepared at different times were the same; there was no evidence of toxicity.

The guinea-pigs that were fed the liquid from the uninoculated tube of beans remained well, but those that were fed from the inoculated tubes died. Those fed from tube 1, that is the tube which had received the heaviest inoculation, died with in 24 hours; the one fed from tube 2 died after 48 hours, and the one fed from tube 3 died after 4 days. There seemed no doubt that the guinea-pigs had died from the ingestion of the toxin of Clostridium botulinum, but to make this more certain, some of the liquid from tube 1 was heated at 100 degrees for 10 minutes. The guinea-pig which received this material remained well, while again the guinea-pigs fed the unheated material died. It is well known that Clostridium botulinum grows readily and forms its toxin in canned string beans. Given a favorable temperature, it appears

to grow equally well in the uncooked beans. String beans contain 50% more protein and 50% more carbohydrate than cabbage. It is possible that the growth which occurs in beans is favored by the amount of protein present. The H-ion concentration of the juice of the two vegetables is about the same. Further experiments are necessary to determine whether in the fermented beans the organism grows only before the material has become distinctly acid. That there was no growth of the organism and merely an increase in the potency of the initial amount of toxin by acidification is quite inconceivable. This might be possible in the tube receiving the heaviest inoculation, but certainly not in the tube receiving the smallest amount. It seems necessary to believe that the toxin in the third tube of beans was the result of growth of the organism, although all such growth may have taken place before the medium became markedly acid, and the potency of the toxin may have been later greatly increased by acidification.

SUMMARY

Clostridium botulinum was found to grow and produce its toxin in fermenting green beans. The results with fermented cabbage were all negative.

PREVALENCE OF TOXIN-PRODUCING ANAEROBES IN WISCONSIN

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Wisconsin has not suffered from poisoning by Clostridium botulinum to any appreciable extent. It is possible that the disease has not been generally recognized until within the last few years, but even with the amount of publicity that has been given of late to outbreaks of botulism in man and animals, there have been no cases of botulism reported from any Wisconsin food product. Two cases of botulism occurred in Milwaukee in 1918,[1] occasioned by eating summer sausage, but in this case the sausage was prepared in another state. From Wisconsin canneries come more than one half of the canned peas used in the United States. Clostridium botulinum grows well in canned peas, and yet no case of botulism has been reported from the use of Wisconsin peas. It is true that peas are usually heated, but they are also used in salads, and if Clostridium botulinum was even infrequently present, some dire results should have followed the general use of these peas.

Forage poisoning seems likewise uncommon among animals in Wisconsin. There have been occasional outbreaks of limberneck or some similar disease in chickens, but no investigations of such outbreaks proving them due to Clostridium botulinum have come to our attention. Thus we have no evidence that botulism has occurred even infrequently in Wisconsin. However, the widely distributed outbreaks of food poisoning of this type in the United States made it seem to us a few years ago that the organism responsible for it was probably present here also and that some investigation as to the frequency of its occurrence should be made. In 1919, one of us (F. B.) isolated an organism from home canned mushrooms which was morphologically and culturally like Clostridium botulinum. This organism when grown in meat produced a toxin which when fed to chickens produced symptoms similar to limberneck. The sterile filtrate from the mushrooms injected intraperitoneally was weakly toxic for guinea-pigs and produced death in one pig after 16 days. The heated filtrate had no effect on the health of the pigs.

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[1] Church, F. E.: Wisconsin Med. Jour., 1920, 18, p. 377.

The soil was chosen as the most likely habitat, but since Burke [2] had found it on the leaves of bush beans, in hog manure and probably in insect droppings, it seemed worth while to include other material than soil as a possible source. The work was begun in the summer of 1920, and the collections of material at that time included considerable other material than soil. The material was collected with aseptic precaution in sterile test tubes or bottles. The following is a list of the material collected at that time: 11 samples of soil from the shore of Lake Mendota, 30 samples from gardens, 1 from a cellar, 9 from roadsides, 10 from grain fields, 4 samples of leaves, 3 flies, 1 spider, 3 samples of fresh spinach, and 1 each of grass, moss, dried rhubarb leaves, and dried tomato plant. Our later collections were almost entirely soil, and were as follows: 21 from gardens, 3 from roads, 3 from chicken yards, 2 from cow lots, 7 from hog lots, 28 samples of clay, sand, loam, and peat soils from different parts of the state, 1 sample of sludge from the Madison sewage disposal plant, 3 samples of hog feces from a packing plant, and 1 sample of soil from an old hog lot. None of these samples was from virgin soil.

We had found that a number of strains of Clostridium botulinum grew very well and produced toxin in sterilized meat and also in canned spinach—hence we used these as medium for our work. Hamburg steak with the addition of enough suet to form a heavy fat layer and enough tap water added to cover the meat and fat was introduced into bottles and sterilized. Likewise fresh spinach was placed in bottles, covered with tap water and sterilized. At first there were duplicate cultures of the spinach and meat but later the use of the spinach was discontinued because the growth in the meat was much more certain. Sterilized bottles of spinach and of meat were inoculated with about 5 gm. of the soil or other material and then heated for 30 minutes at 80 C. or at 100 C. The bottles were incubated at 37 C. for at least 2 weeks, after which tests were made for the presence of toxic substances. This was done by forcibly feeding guinea-pigs 1 to 2 c.c. of the liquid with a pipet. The material was in all stages of decomposition. None of these cultures in the amount fed proved fatal to the guinea-pigs.

Since we had failed to find organisms in any of the material which produced substances sufficiently toxic to be fatal to guinea-pigs, we determined to prove the efficacy of our methods by using material

[2] Jour. Bacteriol., 1919, 4, p. 541.

which was known to contain Clostridium botulinum. To prove that the soil or the microorganisms in the soil did not inhibit the growth of Clostridium botulinum or the production of its toxin, cultures were made of about 5 gm. of soil which had proved nontoxic and inoculated with old pure cultures of Clostridium botulinum which contained largely spores of the organism. Several strains of types A and B of the organisms were used. After incubation of 2 weeks, 1 or 2 c.c. of these cultures with no exception killed guinea-pigs within 24 hours. One of these samples of soil was known to contain B. welchii. To test whether Clostridium botilinum will produce toxin in the presence of other anaerobes, cultures were made using pure cultures of Clostridium botulinium in various combinations with B. sporogenes, B. bifermentans and B. welchii in both meat and spinach. Pure cultures of the different strains of Clostridium botulinum when grown alone in meat and spinach and also when combined with one or more of the above anaerobes were always toxic.

Our failure to obtain a fatal toxic culture from the soil and other materials cultured, and our success in always demonstrating a sufficiently toxic product to be fatal when Clostridium botulinum spores were combined with soil suggested to us that perhaps we might have introduced the organism in larger numbers into the soil than ever occurs naturally, and thereby insured growth, or that the organisms in our cultures were in some condition more favorable for immediate growth than when they were in their natural habitat. Hence it was necessary to convince ourselves further that the method could be relied on. For this reason we obtained, through the kindness of Professor R. Graham of the University of Illinois, 2 samples of material from which he had isolated Clostridium botulinum, namely, a few heads of dried timothy and some canned spinach. This material was introduced into sterile meat in flasks and heated for 30 minutes at 80 C. The flasks then were incubated as above for 2 weeks at 37 C. At the end of this time 2 c.c. from each proved fatal to guinea-pigs in less than 24 hours. From the livers of one of the guinea-pigs which had been killed with the culture of spinach in meat an organism was isolated which had the cultural characteristics of Clostridium botulinum and which readily produced toxin in meat cultures fatal to guinea-pigs.

The method described of obtaining toxin with Clostridium botulinum is very simple. It is possible that some medium such as beef heart peptic digest broth might have given a much more potent toxin. It

will be observed, however, that we readily obtained a toxic product in a meat medium, and were able to isolate the organism from the tissues of an animal killed with it in pure culture when we used material which had been found by Graham to be contaminated with Clostridium botulinum. We also secured toxic products when spores of Clostridium botulinum were mixed with the soil used for inoculating flasks of sterile meat. If very toxic strains were present in any of the material that we examined, there seems to us to be no reason why the organism should not have grown and produced its toxin. Our results do not preclude the possibility that a weak toxin may sometimes have been produced. Occasionally an animal gave some evidence of slight illness, but we chose to regard only those materials as containing Clostridium botulinum which actually killed guinea-pigs when introduced into the meat medium and sufficiently incubated.

Recently Meyer and Dubovsky [3] and their associates have recommended the use of beef heart peptic digest broth for the isolation of Clostridium botulinum and with this medium have been successful in obtaining Clostridium botulinum from the soil in a considerable number of cases. It is probable that such a medium is ideal for the growth of the organism. With the use of this medium they were able to demonstrate weakly toxic strains in 2 out of 36 samples of soil from Wisconsin.[4] Unfortunately, we have not kept records of the guinea-pigs which at times showed some slight illness subsequent to feeding with our soil cultures. It is planned to repeat our studies especially with virgin soils from more extended areas, using beef heart peptic digest broth as well as the simple meat medium above described.

We do not conclude from our results that Clostridium botulinum is absent from the samples of material which we have examined, but rather that it is not abundantly present and certainly that very toxic strains were not present in our samples. Our results are in agreement with the absence of even a questionable occurrence of botulism outbreaks in Wisconsin. Also our findings are in agreement with those of Meyer and Dubovsky showing that occasionally strains of Clostridium botulinum may be found that produce a weak toxin. The extent of these studies is too limited to draw any conclusions as to the general prevalence of the organism in Wisconsin soil.

[3] Jour. Infect. Dis., 1922, 31, p. 501.
[4] Ibid., p. 559.

SUMMARY

One hundred and forty-six samples of Wisconsin soils, leaves, insects, and other material related to soil were cultivated in sterile meat medium to determine whether Clostridium botulinum or other toxin producing anaerobes were present. From home canned mushrooms an organism was isolated which produced symptoms similar to limberneck in chickens, and which was weakly toxic for guinea-pigs. None of the other samples contained organisms which produced substances sufficiently toxic to kill guinea-pigs when these were forcibly fed. Incubation of a few of the samples in the meat medium resulted in the production of substances which were weakly toxic. We conclude that strong toxin-producing anaerobes are not abundantly present in the materials we examined.

COMPLEMENT-FIXATION STUDIES ON CLOSTRIDIUM BOTULINUM *

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An examination of the literature reveals few references to the application of the complement-fixation test to the study of anaerobes; especially is this true with reference to the study of Cl. botulinum. Gaehtgens [1] reports the employment of this test in making a differential study of the organisms associated with gas gangrene. The results obtained were unsatisfactory. McIntosh and Fildes [2] and also Leuchs [3] demonstrated complement-binding bodies in the serum of animals immunized with Cl. welchii. No details are given as to the method employed in immunizing the animals or of carrying out the test. Bitter,[4] in his general discussion of botulism in Europe merely mentions the fact that this type of antibody, as well as agglutinins, is present in the serum of animals that have received injections of the organism. Bronfenbrenner, Schlesinger and Calazans [5] were not successful in using the complement-fixation test for the typing of the organisms, nor for the identification of Cl. botulinum in contaminated material. Kelser [6] reports in detail experiments in which complement fixation was used for the detection of contaminations of canned vegetables with Cl. botulinum. While he was unable to differentiate between the A and the B types by this method, he was successful in demonstrating the presence of the species. There were no nonspecific reactions with normal serum, nor when the vegetables were subjected to mixed contaminations. He used commercial antitoxins as the source of his antibody.

References are occasionally found citing unsuccessful attempts to diagnose suspected cases of botulism by the employment of this test. As the disease is an acute intoxication, such negative findings are to be expected.

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[1] Centralbl. f. Bakteriol., I, O., 1917, 80, p. 166.
[2] Brit. Med. Res. Com., 1917, Ser. 12, p. 74.
[3] Kolle u. Wassermann's Handbuch der Path. Mikroorgan., 1912, 4, p. 944.
[4] Ergebn. d. allg. Path. u. path. Anat., 1921, 19, p. 732.
[5] Proc. Soc. of Exper. Biol. & Med., 1922. 19, p. 21.
[6] Am. Jour. Pub. Health, 1923, 13, p. 366.

Since the results of the work in this laboratory on agglutination reactions with Cl. botulinum[7] showed that specific agglutinins were present in the serum of rabbits injected with the organisms, and that by the agglutination test, not only could the A and the B types of this organism be differentiated but that subgroups within the types could be identified, it was thought advisable to investigate the relation of complement-binding to Cl. botulinum.

1. Are complement-binding antibodies present in the serum of animals injected with Cl. botulinum? If so, do they divide the members into specific subgroups corresponding to those established by the agglutination reaction?

2. Is it possible to use the complement-fixation test, practically, for the identification of Cl. botulinum contaminations in canned vegetables? If so, how early can such contamination be detected, and how long does the evidence of its presence persist?

I. Complement-Fixation in Differentiation of Strains of Cl. Botulinum

Six single cell strains of Cl. botulinum—4 of the A type, and 2 of the B type—were selected for the preparation of antiserums. In order to make more definite comparisons between the 2 kinds of serologic reactions, the cultures chosen were the same as those employed in the work on agglutination. The antiserums were prepared by injecting rabbits with young vegetative cells. The organisms were grown for from 18 to 24 hours at 37 C. in casein digest broth having a P_H of 7.4 to 7.6. The cells were separated from the medium by filtration through sterile cotton and by centrifugation. The sedimented cells were washed from 4 to 6 times in sterile salt solution to free them from any metabolic products or other protein constituents of the medium that might be antigenic and thus interfere with the specificity of the reactions. The organisms were finally suspended in sterile salt solution and heated at 75 C. for 20 minutes to detoxify them. Graduated doses of from 300 to 600 million cells were injected intraperitoneally into rabbits. Although the number of injections varied, a highly potent antiserum was obtained by bleeding the animals from 5 to 7 days after the last of 5 injections, given at 3 to 5 day intervals. The serums thus obtained were inactivated by heating at 53 C. for 20 minutes and were preserved in the refrigerator after the addition of a sufficient quantity of phenol

[7] Jour. Infec. Dis., 1923, 33, p. 169.

to make a 0.5% solution. Immediately prior to being used, the serums were again heated at 53 C. for 15 minutes. This was found necessary with the serums that had been kept for any length of time in order to remove anticomplementary substances that frequently appeared. The hemolytic and the anticomplementary values of the serums were determined before each set of tests, using the method ordinarily employed in the standard complement-fixation technic. Different amounts of serum were used in carrying out the tests in order to obtain quantitative results, but in no instance did the quantity of serum used exceed one half of the anticomplementary value. Normal rabbits furnished the necessary serum controls.

The technic of the test itself varied in no essential from that ordinarily used in bacterial complement-fixation tests. A 5% suspension of well washed red blood cells of the sheep, with 2 units of complement and two units of hemolytic amboceptor constituted the hemolytic system. Titration of the pooled serum from 3 to 5 guinea-pigs was made immediately before each set of tests, to determine the complementary unit. Reagents were adjusted so that 0.25 c c. quantities could be used, and sterile salt solution was added to bring the total volume up to 2 c c. The preliminary incubation for fixation of the complement was 30 minutes at 37 C. in a water bath, with occasional shaking of the racks. The incubation for the determination of hemolysis was carried out in the water bath at 37 C. for one hour. Reading of the results was made after 12 hours' storage in the refrigerator.

Antigens prepared in different ways were tried. These included cultures of Cl. botulinum in medium of various kinds and ages. In general, they were not satisfactory. Young cells washed and resuspended in salt solution gave fairly good results, although the ratio of the antigenic value to the anticomplementary value was small and their successful use required careful standardization. Similar results were obtained when the culture medium was used as antigen. When proper standardization was accomplished, the specificity was lacking. Not only was it impossible with such antigens to confirm the results of the agglutination reactions as regards the subgroups within the types but it was also impossible to differentiate between the A and the B types themselves. The method finally adopted, which gave excellent results, was that developed by Melick[8] in connection with his work on complement-fixation among the aerobes. Young cultures of Cl. botulinum were obtained by growing the organism in casein digest broth for

[8] Jour. Med. Res., 1922, 43, p. 405.

from 24 to 30 hours. Such cultures consisted of practically nothing but vegetative cells. The cells were removed by filtration through sterile cotton and were then centrifuged. They were washed several times in sterile salt solution, and then suspended in sterile distilled water. These suspensions were then subjected to rapid alternations of freezing and thawing, by the use of liquid air and boiling water. It was found that exposure of the cells to the action of the liquid air for from one and one half to two minutes was sufficient to accomplish complete freezing. The number of freezings and thawings varied somewhat, but the average was 10 times with each antigen. This amount of exposure to these extremes of temperature was sufficient to bring about complete disintegration of the cells. The material was centrifuged at high speed until a perfectly clear supernatant was obtained. This was removed carefully, so as not to disturb the sediment, and was recentrifuged. Melick [8] has shown that the decrease in the anticomplementary properties of the antigen depends on the complete removal of all suspended matter. The clear supernatant served as the antigen. It was preserved by the addition of a small quantity of phenol and storage on ice.

The antigens were carefully titrated before using to determine the hemolytic and the anticomplementary values. While variations due to the differences in density of the original suspensions used in their preparation were present, the average anticomplementary value was greater than 1 c c. The hemolytic value was greater than the anticomplementary. As will be noted in table 1, the ratio of the antigenic to the anticomplementary value is extremely large—in some instances being greater than 800.

In carrying out the tests, the antigenic value of each antigen was determined quantitatively in connection with each of the antiserums. Eight different quantities of antigen were used with 2 different amounts of each antiserum, thus giving a quantitative measurement of the complement-binding antibodies present in each serum, and also indicating the degree of specificity of the reaction. The single cell strains of Cl. botulinum used for immunization purposes, and as antigens in the tests were A1, A39, A45, A55, B201, and B207. These strains were representative of the specific subgroups into which 100 single cell strains had fallen, as determined by the agglutination tests.[7]

Each antiserum was tested against both the homologous and heterologous antigens. Table 1 illustrates the methods employed and the results obtained.

TABLE 1

RESULTS OF COMPLEMENT-FIXATION TESTS IN RABBIT SERUM

Number of Rab-	Immunized with	Quantity Serum Used	Antigens Used																	
			A 1			A 45			A 39			A 55			B207			B201		
			0.005 C c	0.0025 C c.	0.00125 C c.	0.005 C c.	0 0025 C c.	0 00125 C c.	0.005 C c.	0.0025 C c.	0.00125 C c.	0.005 C c.	0.0025 C c.	0.00125 C c.	0.005 C c.	0.0025 C c.	0.00125 C c.	0.005 C c.	0.0025 C c.	0.00125 C c.
82	A 1	0.025	++++	++++	++++	++	+	0	+	±	0	0	0	0	0	0	0	0	0	0
		0.0125	++++	++++	++++	±	0	0	+	0	0	0	0	0	0	0	0	0	0	0
150	A 45	0.025	++	+	0	++++	++++	++++	++	+	0	0	0	0	0	0	0	0	0	0
		0.0125	+	0	0	++++	++++	+++	0	0	0	0	0	0	0	0	0	0	0	0
283	A 39	0.025	++	+	0	++	+	0	++++	++++	++++	0	0	0	0	0	0	0	0	0
		0.0125	0	0	0	0	tr	0	++++	++++	++++	0	0	0	0	0	0	0	0	0
5	A 55	0.025	0	0	0	tr	0	0	0	0	0	++++	++++	++++	+	0	0	0	0	0
		0.0125	0	0	0	0	0	0	0	0	0	++++	++++	++++	0	0	0	0	0	0
281	B207	0.025	0	0	0	0	0	0	0	0	0	0	0	0	++++	++++	++++	0	0	0
		0.0125	0	0	0	0	0	0	0	0	0	0	0	0	++++	++++	++++	0	0	0
133	B201	0.025	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++++	++++	++++
		0.0125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++++	++++	++++
N/1	....	0.025	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0.0125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

++++, +++, ++, + indicate decreasing degrees of fixation.
"0" indicates complete hemolysis.

A study of table 1 shows a definite differentiation between the A and B types of Cl. botulinum. While fixation occurred with the use of much smaller quantities of antigen than are indicated in the table—even as low as one half to one fourth of the amount given—such reactions were incomplete and are omitted. When larger amounts of the antigen were used, e. g., 0.02 to 0.05 c c., partial bindings were obtained with the heterologous antiserums. However, the quantitative differences were so marked as to eliminate any confusion in determining the relationship. The tendency to cross reactions was more marked with the B than with the A types. Especially was this true with B207. Within the 2 types, the division into subgroups was also evident, though here the differences are quantitative. While there was partial binding with different antigens when the larger quantities were used in conjunction with a larger volume of the serum, the quantitative tests also definitely differentiated the subgroups. The subdivisions are identical with those obtained by the agglutination reactions.[8]

The same peculiar relationship as regards A55 is evident in the complement-fixation test, as was noted in the agglutination test. This strain, while producing a highly potent toxin which is neutralized by the A antitoxin, shows no closer relation to the other members of type A than do the B strains. However, it is interesting to note that while this organism was nonantigenic in the agglutination work—neither stimulating the production of agglutinins, nor being agglutinated by any antiserum—there were demonstrable specific complement-binding bodies in the serum of animals immunized with A55.

In addition to the organisms listed in table 1, eight other strains, whose grouping had been determined by the agglutination tests, were selected, and antigens were prepared in a similar manner. They were then given arbitrary numbers and tested as unknowns. In no instance was any difficulty encountered in determining the types of organisms, or in placing them in their proper subgroups.

SUMMARY

Using as antigen single cell strains of Cl. botulinum whose cells had been dissolved by alternate freezing and thawing, it was possible to demonstrate the presence of complement-binding bodies in the serum of animals immunized with these same strains. The reactions were specific as regards the A and the B types of Cl. botulinum, and by employing quantitative methods it was possible to demonstrate sub-

groups within the types. The 4 subgroups within type A and the 3 within type B correspond to the subgroups determined by agglutination reactions.

II. THE DETECTION OF CL. BOTULINUM CONTAMINATION IN CANNED VEGETABLES BY MEANS OF THE COMPLEMENT-FIXATION TEST

During the early part of the work on complement-fixation in its relation to the identification of types and strains of Cl. botulinum, a few attempts were made to demonstrate complement-binding bodies in specific antiserums by using as antigens extracts of vegetables that had been inoculated with different single cell strains. The number of these attempts were few, the results inconstant, and nonspecific reactions frequent. Because of the unsatisfactory results, these attempts were discontinued at that time.

The practical value of such a procedure for the identification of contaminations in foodstuffs is evident.

Shortly subsequent to this time, Kelser [6] reported successful results in detecting such contaminations, using ordinary antitoxic serums as the source of the antibody, and various preparations of canned vegetables as antigens. It was decided to take up this line of work again to determine whether the findings of Kelser could be confirmed, and in addition to gain some information as to the time of the appearance and the persistence of these antigenic constituents in the contaminated materials.

The Antiserums.—As a source of the complement-binding antibodies, 5 serums were used. Four of these—2 A types and 2 B types—were antitoxic serums obtained from different laboratories. The other antiserum was one obtained from rabbits that had been artificially immunized with one of our single cell strains of Cl. botulinum. This antiserum was prepared against the A1 strain, and was specific for this subgroup. Serum from normal rabbits served as controls. Since the antitoxic serums were highly anticomplementary, they were heated at 53 C. for 20 minutes just before being used. The maximum quantity of antiserum used in the tests was never greater than one half of its anticomplementary value.

The Antigens.—A total of 25 different antigens were used in the course of the work. The mediums inoculated consisted of peas, corn and spinach; beef heart medium and casein digest broth were also used in a few instances for comparative purposes. The cultures used for

inoculation of these mediums were A1, A39, A45, A55, B201, and B207. Cl. sporogenes and Cl. putrificum were also inoculated into the same kind of mediums to serve as controls on specificity.

The following methods of preparing the antigens, as suggested by Kelser, were employed:

1. The inoculated medium was centrifuged. . In some instances, when little fluid was available, the vegetables—prior to centrifuging—were ground in a sterile mortar with sterile sand and a minimum quantity of salt solution. The supernatant fluid was transferred to sterile tubes and heated in the autoclave, at 12 pounds' pressure, for 15 minutes. Any precipitate appearing as the result of the heating was removed by centrifuging, and this clear fluid served as the antigen.

2. The inoculated medium was centrifuged and the supernatant used without further treatment.

3. The medium was ground in a mortar with sterile salt solution and evaporated to dryness on a water bath. The residue was resuspended in sterile salt solution and centrifuged. The supernatant served as the antigen.

4. Old cultures containing practically nothing but spores were centrifuged, the supernatant discarded, and the spores washed from 2 to 4 times in sterile salt solution. They were then heated in the autoclave, at 12 pounds' pressure, for 15 minutes, and recentrifuged.

These stock antigens were diluted 1 : 3 with sterile salt solution before titration.

The inoculated mediums were incubated for the following periods of time: 60 hours at 37 C., and 6 weeks, 8 weeks, and 7½ months at room temperature. The cultures were all toxic at the time the antigens were prepared, as was shown by the injection of small quantities into white mice, with and without the homologous antitoxins. No attempts were made, in this work, to use mixed culture antigens, and the cultures were always tested for the presence of aerobic contaminations before being used. The anticomplementary and the hemolytic values of the antigens were determined in the usual manner. The anticomplementary action of these antigens was marked, and difficulty was encountered in carrying out the tests because of the relation existing between this value and the antigenic value of the antigen. While this range varied somewhat with the different methods of preparation, it was always narrow—the ratio between the two being less than 15 in most instances. The hemolytic action was also marked, but since this

TABLE 2

COMPLEMENT-FIXATION TESTS OF ARTIFICIALLY CONTAMINATED VEGETABLES

Antigen						Results—Serum Used			
No.	Medium	Inoculated with	Incubation Period	Method of Preparation	Quantity	No. 12	N. Y. A.	J. S. B.	N. Y. B.
1	Corn.....	Cl. sp.	60 hrs.	1	0.05	0	0	0	—
					0.025	0	0	0	—
					0.01	0	0	0	—
2	Peas.....	A 1	60 hrs.	1	0.15	++++	++++	++++	—
					0.10	+++	++++	+++	—
					0.05	+	+++	+	—
3	Corn.....	uninoculated	60 hrs.	—	0.15	+	+	+	—
					0.10	0	0	0	—
					0.05	0	0	0	—
4	Spinach.....	A 1	60 hrs.	1	0.15	++++	++++	+++	—
					0.10	++++	++++	+++	—
					0.05	++++	++	++	—
5	Spinach.....	uninoculated	60 hrs.	—	0.15	0	+	+	—
					0.10	0	0	0	—
					0.05	0	0	0	—
6	Corn.....	B201	60 hrs.	1	0.15	—	++++	++++	++++
					0.10	—	++++	++++	++++
					0.05	—	++++	++++	++++
7	Peas.....	uninoculated	60 hrs.	—	0.15	0	—	0	—
					0.10	0	0	0	—
					0.05	0	0	0	—
8	Spinach.....	B201	60 hrs.	1	0.15	++++	++++	++	—
					0.10	+	+++	++	—
					0.05	+	+++	++	—
9	Corn.....	A 1	60 hrs.	1	0.15	++++	++++	++	—
					0.10	+++	++++	+	—
					0.05	+	++	+	—
10	Peas.....	B201	60 hrs.	1	0.15	—	++++	++++	++++
					0.10	—	++++	++++	++++
					0.05	—	++++	++++	++++
11	B. H. M.	A 1	8 days	1	0.05	++++	++++	++++	—
					0.025	++++	++++	++++	—
					0.01	++++	++++	++++	—
12	Peas.....	A 1	8 days	2	0.05	++++	++++	++++	—
					0.025	++++	++++	++++	—
					0.01	++++	++++	++++	—
13	Spinach.....	A 1	8 days	3	0.15	++++	++++	++++	—
					0.10	++++	++++	++++	—
					0.05	++++	++++	++++	—
14	Corn.....	B207	8 days	3	0.15	++++	—	++++	++++
					0.10	+++	—	++++	++++
					0.05	++	—	++++	+++
15	Corn.....	B201	8 days	1	0.15	+++	—	++++	++++
					0.10	+++	—	++++	++++
					0.05	++	—	++++	+++
16	Peas.....	A 39	8 days	1	0.15	++++	++++	++++	—
					0.10	++	++++	+++	—
					0.05	0	++++	+++	—
17	Corn.....	B201	6 weeks	1	0.05	+++	—	++++	++++
					0.025	++	—	++++	++++
					0.01	++	—	++++	++++
18	B. H. M.	A 55	6 weeks	1	0.10	0	++++	++++	—
					0.05	++	++++	++++	—
					0.025	+	++++	++++	—
19	Corn.....	B201	6 weeks	4	0.20	++++	—	++++	++++
					0.10	+++	—	++++	++++
					0.05	+++	—	++++	++++
20	Peas.....	B201	6 weeks	1	0.20	++++	—	++++	++++
					0.15	+++	—	++++	++++
					0.10	+++	—	++++	++++
21	Casein.....	A 39	8 weeks	4	0.20	++++	++++	++++	—
					0.10	+++	++++	++++	—
					0.05	+++	++++	++	—
22	Casein.....	B207	8 weeks	1	0.20	++++	—	++++	++++
					0.10	+++	—	++++	++++
					0.05	++	—	++++	++++
23	Corn.....	A 1	8 weeks	1	0.15	0	+++	++++	—
					0.10	0	+++	++++	—
					0.05	0	+++	++++	—
24	Peas.....	Cl. put.	8 weeks	1	0.15	0	0	0	—
					0.10	0	0	0	—
					0.05	0	0	0	—
25	B. H. M.	A 55	7 months	1	0.05	++++	++++	++++	—
					0.025	++++	++++	++++	—
					0.01	++++	++++	++++	—

++++, +++, ++, + indicate decreasing degrees of fixation; "0" indicates complete hemolysis; "—" indicates that the particular serum was not used.

1, 2, 3, 4 refer to the different methods of preparing the antigen, as described in the text.

"N. Y. A." is a commercial antitoxin of the A type; "J. S. B." and "N. Y. B." are commercial antitoxins of the B type; "No. 12" is an antiserum prepared by immunizing rabbits with pure cultures of A.1 strain of Cl. botulinum.

All antigens listed in the chart were run against normal rabbit serum in the following quantities—0.20, 0.15, and 0.10 c c. In no case was there any binding.

was always greater than the anticomplementary, it interfered in no way with the performance of the test.

The Test.—The technic of the test, as regards time and temperature of incubation, and the hemolytic system employed, was the same as that described in the first part of this paper. Three quantities of antigen were used to make the test relatively quantitative. The maximum amounts of the antigens used varied between one third and one sixth of their anticomplementary values. The serum and the antigen controls duplicated the quantities used in the test proper. Uninoculated vegetables treated in the same manner as regards incubation and the preparation of the antigen were carried through as controls on the binding properties of the medium. Antigens prepared from cultures of Cl. sporogenes and Cl. putrificum served as controls on the specificity of the reaction with reference to other anaerobes.

SUMMARY

Examination of the results obtained shows that in vegetables inoculated with Cl. botulinum, antigenic substances are produced that are capable of binding complement in the presence of ordinary botulinus antitoxin, as well as of an antiserum produced by the artificial immunization with bacterial cells. The presence of these substances can be detected within 60 hours after inoculation, when the cultures have been incubated at 37 C., although at this time the results are variable, and in some instances only partial fixation is obtained. Vegetables tested 8 days after inoculation gave positive results, and the antigenic principle was still present in vegetables after 8 weeks, and in beef heart medium after 7½ months. As regards lack of specificity, the results are in accord with those of Kelser. No differentiation between A and B types was possible. This is particularly interesting with Serum 12, which was specific for a particular subgroup of type A, when tested with an antigen prepared by lysing the washed cells of the organism. Evidently the antigenic substance in the culture medium, including, as it must, the metabolic products of the growth of the organism, is not identical with that found in the cells themselves.

While the contamination of vegetables inoculated with Cl. botulinum was successfully demonstrated, considerable difficulty was encountered in carrying out the tests, due to the occurrence of nonspecific reactions. The ratio between the antigenic and anticomplementary values of the antigens is so small that unless strict attention is paid to the standardiza-

tion of all the reagents, and every factor is carefully controled, nonspecific reactions will occur frequently, and doubts arise as regards the reliability of the test.

Conclusions

The injection of single cell strains of Cl. botulinum into rabbits stimulates the production of specific complement-binding antibodies.

The subjection of well washed vegetative cells of Cl. botulinum to alternate freezing and thawing produces an antigen which is highly satisfactory. It exhibits practically no anticomplementary action, and the antigenic value is high.

Utilizing this antigen with antiserums prepared by the injection of single cell strains makes it possible to separate Cl. botulinum into A and B types which are analogous to the types based on antitoxins. Subgroups within the types are also demonstrable by this method—4 within the A and 3 within the B type—and these subgroups are identical with those determined by the agglutination reaction.

Cl. botulinum inoculated into peas, corn, and spinach, gives rise to antigenic substances that are detectable by the complement-fixation reaction when tested with botulinus antiserums.

Four commercial antitoxins contained the specific antibodies, as did also the antiserum prepared by immunizing rabbits with pure cultures of the organism.

The antigenic substances are demonstrable within 60 hours after inoculation, and persist for at least 8 weeks in vegetables kept at room temperature.

The specificity of the reaction when the antigens are derived from inoculated vegetables is much less than when they are prepared from cultures of the organisms—it being impossible in the former instance to differentiate between the A and B types of Cl. botulinum. It is also true that the ratio between the antigenic and the anticomplementary values of such vegetable antigens is small, and very careful titration of all reagents is necessary in order to avoid nonspecific reactions.

Vegetables inoculated with Cl. sporogenes and Cl. putrificum did not yield positive results when tested against botulinus antiserum.

PURE CULTURES OF CL. BOTULINUM FROM SINGLE CELLS

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One of the striking features in the study of anaerobes is the diversity of descriptions of cultural and serologic characteristics of the organisms. The descriptions are often so contradictory that identification of particular organisms is impossible. Without doubt one of the chief causes of this confusion is the use of impure cultures. All workers with anaerobes agree that the problem of obtaining pure cultures of such organisms is much more difficult than in the study of aerobes. The greater tendency toward a symbiotic existence, the absence of the clear-cut differential reactions, and the greater technical difficulties involved, are some of the factors contributing to the difficulty. No description of the methods employed for the isolation of anaerobes by the different workers will be given here, since many excellent reviews are available.[1] There is no doubt of the value of these methods, and by their employment alone pure cultures can be obtained. However, they generally consume much time, require repeated applications, and in the end there is no certainty that adventitious organisms, which may later develop and result in mixed cultures, are not present.

Mechanical isolation of single cells under direct microscopic observation has been employed successfully in the study of aerobes for some time. The application of similar methods to the isolation of anaerobes has been limited. Tulloch,[1] Heller,[1] Hall,[1] and Bengtson [1] report the use of this method, although generally the results were considered unsatisfactory, and the method was discarded. Barber [1] (1920) reports the successful isolation of single cells from 15 different anaerobes,

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[1] Barber, M. A.: Philippine Jour. Sc., Series B, 1914, 9, p. 307; Jour. Exper. Med., 1920, 32, p. 295. Barnard and Morgan: Proc. Roy. Soc. Med., 1903, 72, p. 126. Bengtson, Ida A.: Pub. Health Rept., 1922, 37, p. 2252. Chambers, R.: Jour. Infect. Dis., 1922, 31, p. 334; Anat. Rec., 1922, 24, p. 1. Hall, I. C.: Univ. California Publications in Path., 1915, 2, p. 147; Jour. Infect. Dis., 1922, 30, p. 445. Hecker, F.: Ibid., 1916, 19, p. 306. Heller, H. H.: Jour. Bacteriol., 1921, 6, p. 445. Henry, H.: Jour. Path. and Bacteriol., 1916, 21, p. 344. Hort, E. C.: Jour. Hygiene, 1919, 18, p. 361. Jorgensen: Micro-Organisms and Fermentation, 1911. Kahn, M. C.; Jour. Med. Res., 1922, 43, p. 155. Kendall, Cook and Ryan: Jour. Infect. Dis., 1921, 29, p. 227. Mutch, N.; Jour. Roy. Micro. Soc., 1919, 11, p. 221. Schouten, S. L.: Ztschr. f. Wissensch. Mikr., 1905, 22, p. 10. Sturges and Rettger: Jour. Bacteriol., 1919, 4, p. 171. Topley, Barnard and Wilson: Jour. Hygiene, 1921, 20, p. 221. Tulloch, W. J.: Jour. Roy. Army Med. Corps, 1917, 29, p. 631; Brit. Med. Jour., 1918, 1, p. 614.

with a relatively high proportion of growths. He is of the opinion that the method is "feasible for this purpose." Kendall, Cook, and Ryan,[1] and Kahn,[1] using a slightly modified technic, report successful results. Theoretically, the method should furnish the most definite means of securing pure cultures. The reported technical difficulties, and the small percentage of growths obtained from isolated cells, have restrained many from undertaking this line of work.

Of the various methods suggested for the isolation of single cells, that of Barber, as originated by him, or as slightly modified by Chambers,[1] has been most commonly used, and has yielded most satisfactory results. The literature dealing with the various methods of single cell isolation is quite extensive. It has been reviewed recently by Barber,[1] Chambers,[1] and Hort,[1] to whose articles the reader is referred.

As an initial step in the study of Cl. botulinum, the isolation of single cells of the organism was undertaken, not only to secure pure cultures, but also with the hope of acquiring some knowledge of the resistance of the organism, and of the conditions of growth and toxin production.

TECHNIC

Generally speaking, the method employed was that of Barber; some minor modifications suggested themselves during the course of the work. In the isolation of single bacterial cells by the capillary pipet method, the proper preparation of the cover glass on which the isolations are to be made, and the proper preparation of the capillary pipet are the most important and most difficult steps in the procedure. With these two instruments correctly prepared, the actual isolation is comparatively simple. The slight modifications of Barber's technic seemed to make these manipulations easier and to yield more nearly constant results.

The isolation chamber is prepared by cementing ordinary microscopic slides together with a mixture of Canada balsam and sealing wax. The dimensions of the box are similar to those used by Barber, but instead of leaving the entire top open, thus requiring a large cover glass (60 x 35 mm.), the top is partly closed by a glass slide so cut as to leave an opening which can be covered by a 22 x 22 mm. glass. Such covers are much more easily obtained, are less expensive, and in addition are much more easily cleaned. The top, ends, and sides of the box are lined with ordinary filter paper, which is moistened with water just before isolations are made, thus reducing to a minimum the difficulty caused by the drying of the droplets.

The cover glasses are washed thoroughly in a hot soap solution, heated in a hot bichromate cleaning mixture, rinsed, and then boiled in several changes of distilled water and dried with a grease-free cloth. Such a cloth is prepared by washing a good grade of cheese cloth, or light weight muslin, in several changes of water to which Na_2CO_3 has been added, rinsing in distilled water and drying. The cover glasses are then placed in Petri dishes and heated in a hot air sterilizer at a temperature sufficiently high to volatilize any remaining

traces of grease. When a cover glass is to be used, a mere trace of sterilized petrolatum is touched to the side and evenly distributed over the surface; the fingers are then moistened with soap solution and the surfaces thoroughly rubbed, after which the glass is washed in ordinary distilled water. Hot water was found not to be so satisfactory, since it removed too much grease, thus allowing the droplets to spread too much. The covers are again dried with a grease-free cloth, and passed quickly through a flame to produce a smooth uniform film of fat, and to sterilize the surface. By careful observation of these details, the difficulty of securing satisfactory covers is practically eliminated.

Whether the isolation of single cells is easy or is impossible depends in large measure on the character of the capillary pipet. The tip of a pipet for isolation purposes should be not more than 3 to 5 microns in diameter. For the subsequent transfer of the isolated cells, pipets with a larger bore may be used. To prepare the pipets, sterile glass tubing of about 1.5-2 mm. in diameter should be drawn out quickly in a blast flame, making a very small bored tube. This is again drawn out in a micro-burner with a pin point flame, to produce a sealed capillary tip. The tip is turned to form a right angle with the tube by careful softening over the micro-burner. The pipet is then placed in the holder. Attempts to break the closed tip by contact with the under surface of the cover were not very successful on account of the extreme flexibility of the glass, and also because of the danger of its contamination by touching inoculated droplets. A method which has proved quite satisfactory is to clip the tip of the capillary pipet with small sterile scissors under low power of the microscope. After some practice, there is little difficulty in securing tips of any desired caliber. Control tests on empty pipets prepared in this manner invariably demonstrated their sterility.

The following technic of isolation was used throughout the work. Place a series of small sterile drops of a perfectly clear medium on the prepared cover glass. One or more of these droplets are inoculated with material from the culture from which isolations are to be made. The capillary pipet, already in position, is filled by capillarity from one of the sterile supply drops, and then a few organisms allowed to enter from an inoculated droplet. The box is then moved to expose a free space on the cover glass, and under microscopic control, a series of single cells are discharged into small droplets. This is satisfactorily done under the high dry objective. The drops are then carefully examined to insure the presence of single organisms only, and to note the characteristics of the isolated cell. Satisfactory droplets are then carefully charted. With properly prepared covers, and a capillary pipet of suitable bore, there is no difficulty in ascertaining the presence of a single organism. The only explanation of Hort's criticism relative to this point must lie in the character of the cover or in the size of the pipet used by him.

The isolated cell is then taken up in a new sterile capillary pipet, and transferred to the desired culture medium. These pipets need not possess so fine a capillary tip, and are made up in quantity and kept in sterile containers until needed. The time of exposure of the cell is of little importance, as the transfer can be accomplished quickly. In expelling the cells from the transfer pipet into the culture medium, the discharge is continued until a few air bubbles appear. The pipet is then washed by repeatedly drawing in and expelling the liquid medium. While there can be no absolute knowledge that the isolated cell has been discharged, control manipulations under microscopic observation show that the cells pass from the pipet readily, and it is probable that the number of negative results due to failure to expel the cell is small.

STRAINS OF CL. BOTULINUM

Thirty-three strains, purified by ordinary bacteriologic methods, were received for study. Eight were furnished by Dr. J. C. Geiger; 1 by the Ohio State Department of Health; 1 by Dr. W. G. Sackett of Colorado; 11 by Dr. K. F. Meyer of the University of California; 11 by Dr. R. Graham of the University of Illinois; 1 by Miss Bengtson of the Hygienic Laboratory. In this group of strains, there were 21 of type A, 11 of type B, and one of type C. These cultures were all tested for specific toxin production, and for aerobic contaminations. All conformed to the indicated type when tested with specific antitoxin, and were free from aerobic contaminants. In a few instances, anaerobes other than Cl. botulinum were isolated from these stocks.

STRAINS AFTER ISOLATION

Subcultures were made of the organisms selected for isolation. At first, beef heart medium was employed, but was soon discarded because of the difficulty of differentiating free spores from the highly refractive fat globules of the medium. A perfectly clear medium is an absolute essential for satisfactory isolation of spores. The most satisfactory medium when spores were desired proved to be a pea juice gelatin;[2] coagulated egg albumin broth or Wolff's casein digest broth served best when vegetative cells were desired. Incubation at 37 C. for ten to fourteen days yielded most satisfactory free spores. Spores within the cells were abundant after 4 to 6 days' incubation in this pea juice gelatin; young 18- to 24-hour cultures incubated at 37 C. yielded the best vegetative cells. Ordinarily no preliminary dilution of the culture was necessary. The small droplets of sterile broth on the cover were inoculated with a fine straight transfer needle, and sufficed to yield a suitable dilution. The different cultures varied somewhat in character of growth. In most instances the cells occurred singly, but in certain strains they showed a tendency to develop in diplo-grouping, or even in sheet filaments. In these cases, the filamentous growths were considered as individuals, and isolations were made of the same. It is evident that this would introduce no error as regards the purity of the cultures obtained, while increasing the chances of securing growth. In the isolation of single cells, or of spores within the cell, an attempt was always made to select only such as were motile; when the technic of isolation was perfected, such cells as a rule were still exhibiting motility on introduction into the tube, thus insuring the transfer of a living cell. It is evident that no such criterion could be established for free spores.

CULTURE MEDIUMS

Glucose broth, glucose semisolid agar, and pea juice gelatin were most commonly employed for the growth of the isolated cells. Glucose agar, brain medium, and cooked beef heart medium were used in a limited number of instances. Veal infusion served as the basis of all meat extract mediums; 0.25% glucose was used in all sugar mediums; 0.50% gelatin was used in the pea juice medium; and 0.25% agar was used in the semisolid agar medium. The reaction was adjusted to P_H 7.4 to 7.6 after sterilization.

The sterility was assured by preliminary incubation at 37 C., for 4 days, after which time transfers were made to agar slopes and beef heart medium from 10 tubes selected at random from a particular lot of medium. These were incubated for another week at 37 C. before any of the medium was used.

[2] Dubovsky and Meyer: Jour. Infect. Dis., 1922, 31, p. 505.

Just before the introduction of the organism, every medium used for cultivation of the isolated cells was boiled at least 15 minutes to expel the oxygen, and cooled quickly by immersing in ice water. Anaerobiosis was maintained by covering the medium with a layer of sterile petrolatum 1.5 cm. to 2.0 cm. deep. This seal was made immediately after the introduction of the organism, thus avoiding the danger of losing the cell while passing through the petrolatum.

All single cell isolations showing indications of growth were examined to determine the purity of the culture. This examination consisted of a morphologic study of the organism in both young and old cultures. Gram's stains were made; motility was determined by hanging drop examinations, and the absence of aerobic contaminants determined by the inoculations of large quantities of the culture to agar slopes by means of Pasteur pipets. After 10 days of growth, the ability of the organism to produce botulinus toxin was determined by injections into mice, controlling with the use of antitoxins A and B.

RESULTS OF SINGLE CELL ISOLATION IN CULTURE TUBES

Both vegetative cells and spores were isolated. Under the term "spores" are included those forms still within the cell, as well as those that were free. The term "vegetative cells" includes a few filaments

TABLE 1

VARIATIONS IN THE NUMBER OF POSITIVE GROWTHS FROM 10 REPRESENTATIVE STRAINS

Strains	Number of Isolations	Number of Growths	Percentage Growths
A 8	43	8	16.3
A 3	29	8	27.6
B 6	22	15	68.1
A 5	32	6	18.75
D I	40	7	17.5
B2HB	32	12	37.5
276B	25	10	40.0
M 4	15	1	6.6
M 7	15	5	33.3
KIA	30	13	43.3

composed of several cells, but considered as a unit. Seven hundred and fifty-two isolations into culture tubes were made from the 33 strains of Cl. botulinum; 227 growths (30.2%) resulted. The number of isolations made from the individual strains varied from 10 to 40. Marked variation was observed in the proportionate number of growths obtained from the different strains, although care was exercised to maintain constant conditions throughout. In certain strains the percentage of positive growths was as high as 60%; in others, only one or two growths were obtained from 20 isolations; from one strain only (Bengtson's type C) no growths were obtained, although 30 isolations were made. No explanation of this variation is available.

While considerable variation was noticed among the different strains as regards the percentage of growths resulting from the isola-

tion of single cells and spores, respectively, the general proportion of growths from spores was appreciably higher. Of the 752 isolations made, 374 were spores, yielding 137 growths (36.6%); from the 378 vegetative cells isolated, there were 90 growths (23.8%).

Table 2, which gives the results of the inoculation of isolated spores and cells into different mediums, indicates no definite superiority of any one medium. When spores were inoculated, the semisolid agar gave somewhat better results than the other mediums; in the case of vegetative cells, glucose broth yielded a slightly higher percentage of positive results. The number of isolations transferred to the other mediums is too small to justify any conclusions.

TABLE 2

PROPORTION OF GROWTH IN VARIOUS MEDIUMS

Medium	Total Spores	Total Growth	Percentage Total	Total Vegetative Cells	Total Growth	Percentage Total
Glucose broth	163	52	31.9	156	42	26.8
Glucose semisolid agar	126	51	40.5	137	29	21.2
Pea juice gelatin	69	26	37.7	67	15	22.4
Beef heart	8	5	....	9	3	....
Brain	8	3	....	9	1	....

EFFECT OF EXPOSURE TO AIR ON CELLS

The intervals of time during which cells were exposed to air during the isolations were noted. This time was calculated from the moment the material was removed from the stock culture until the isolated cell had been introduced into the medium in which it was to be incubated. The character of the isolated cell was also noted, thus making it possible to arrive at some conclusion as to the effect of exposure on the ability of the spore and the vegetative cell to grow. Table 3 shows the results obtained.

TABLE 3

RELATION OF TIME OF EXPOSURE TO GROWTH OF SPORES AND CELLS

Spore Isolations				Vegetative Cell Isolations			
Time of Isolation, Minutes	Total Number	Number of Growths	Percentage of Growth	Time of Isolation, Minutes	Total Number	Number of Growths	Percentage of Growth
4 - 6	42	14	33.3	4 - 6	57	11	20
7 - 10	158	66	41.75	7 - 10	186	45	24
11 - 14	93	36	38.5	11 - 14	89	19	21
15 - 20	62	27	43.5	15 - 20	41	8	19.5
20 -	1	0	....	20 -	1	0	....
	356	143			374	83	

Table 3 shows no apparent relation between time of exposure and the percentage of growth among spores when the time of isolation is under 20 minutes. In the case of vegetative cells, there is a tendency toward a slight decrease in the proportion of positive results as the time of exposure is increased beyond 10 minutes. Since the primary object of this work was to secure a sufficient number of single celled cultures for subsequent needs, and not to make an exhaustive study of all the factors involved, no attempt was made to determine the maximum exposure that would be tolerated by spores and vegetative cells. The general rule was established to discard all cells in which the period of isolation exceeded 20 minutes. Fifteen per cent. of the isolations, with 11.5% of the growths, required only 6 minutes or less; in 60.5% of the isolations, with 60% of the growths, the time of isolation was not more than 10 minutes. It is evident that with some practice in single cell technic, the time of exposure necessary in the isolation of anaerobes need not be so long as to be a factor in limiting the number of growths obtained.

EFFECT OF SYMBIOSIS ON THE PROPORTION OF GROWTHS

In addition to the 752 isolations previously mentioned, a series of isolations were made in conjunction with some nonsporulating aerobes. The organisms used were B. coli, B. fecalis alkaligines and Ps. pyocyanea. Mass inoculations of the aerobe used in each instance were made ordinary loop transfer. The number of cells introduced was varied by dilutions of the stock. The inoculations with the symbiont were made at the time the isolated cells were introduced. The presence of the aerobe alone was depended on to establish anaerobiosis and no petrolatum seal was employed. After microscopic examination revealed the presence of sporulating Cl. botulinum, the elimination of the aerobe was successfully accomplished by heating the cultures at 75 C. for 30 minutes.

Fifty such isolations were made. The number of positive growths obtained was 16 (32%), which is only slightly greater than was obtained when the symbiont was omitted. Since this technic involved the additional step of the removal of the aerobe, it was discarded. When the second organism was used, the time intervening before the appearance of growth of Cl. botulinum was somewhat shortened. Of these 16 growths 12 appeared within 2 days, 2 within 4 days, and 2 others within 5 days. There was no development of additional single cell isolations, although they were kept under observation for more than 2 months.

EFFECT OF THE INCUBATION TEMPERATURE ON GROWTH

The question as to what constitutes the optimum temperature for the growth and toxin production of Cl. botulinum has been a much disputed one. The earlier workers reported 18 C. to 20 C. as the optimum, some even denying the ability of the organism to grow at 37 C. Other investigators admitted growth, but denied the ability of the organism to produce toxin at the higher temperature. In view of this difference of opinion, isolations were incubated at room temperature (20 C. to 22 C.) and at 37 C. The results are in agreement with the findings of later workers, viz., that not only is there satisfactory growth at 37 C., but that equally good results as regards toxin production are obtained when incubation is carried on at 37 C. as at room temperature. Of 558 isolations, 361 were incubated at 37 C. and yielded 112 growths (31%); 197 were incubated at room temperature and yielded 63 growths (32%). The period of time before the appearance of growth averaged much less among those isolations incubated at 37 C. than among those kept at room temperature. The rate of toxin production was also much greater at the higher temperature, although there was no difference observed in the amount of toxin eventually produced.

TIME OF APPEARANCE OF GROWTH

Accurate records of all isolated cultures were kept to determine the length of time necessary for growth to manifest itself. The criterion of growth was the clouding of the culture medium with the first appearance of gas. Considerable variation was found. Generally speaking, the time before the appearance of growth when the isolated cell was in the vegetative state was shorter than when in the spore stage, although this difference was not marked or constant. Of 137 spore isolations that grew, 80, or 58.4%, manifested growth within 7 days; of 90 vegetative cell isolations resulting in growth, 51, or 56.6%, grew within 7 days. The earliest appearances of growth were evident after 48 hours (9% of the spore isolations, and 10% of the vegetative cell isolations). Twenty-seven spores (18.6%) and 9 vegetative cells (10.7%) showed growth only after 3 weeks' incubation. In a few instances, the time was much greater, e.g, 3 spore isolations showed growth only after 55 days; 2 after 60 days; and in one instance, the first evidence of growth appeared after 90 days. The cultures were kept under observation for 8 months, but no further growths developed. Morphologically and culturally these delayed growths checked with Cl.

botulinum, and produced the characteristic type toxin. No appreciable variations existed among the cells of the different strains, nor was there any difference noted between the rates of development of type A and type B strains. The rate of toxin production also was noted. Toxin production was apparently a function of the rate of growth, and the time of maximum toxin content after the first indications of growth was no different from that observed when mass inoculations were made into the same kind of mediums and kept under similar conditions. As a matter of routine, the presence of the toxin was determined by intraperitoneal injections into mice 5 to 7 days after growth appeared.

OTHER METHODS OF ISOLATION

In addition to the methods of isolation described, attempts were made in a limited number of instances to grow the isolated cells in the hanging drop: sometimes by the employment of chemical reducing agents, as alkaline pyrogallic acid placed in the deep well of the hanging drop stage; sometimes by cultivating the cell in symbiosis with non-spore-bearing aerobes, grown in the well of the slide, e.g., Ps. pyocyanea or B. coli. The use of chemical reducing agents failed to yield a single positive result in 50 isolations. The employment of the aerobic symbionts yielded 10 growths from 50 isolations (20%). This was about 10% less than was obtained by the culture tube method, and since the labor involved was greater, including as it did the subsequent elimination of the aerobe, and also since the possibility of contamination was increased by the additional manipulations, the method was abandoned.

DISCUSSION

The isolation of 800 single cells, with 253 successful growths, indicates the practicability of the method for securing pure cultures of anaerobes. While the technic is exacting, it is not difficult to acquire, and when once mastered makes possible the isolation of pure cultures more rapidly than by the other ordinary methods. During the later part of the work, 60 isolations, yielding on an average of 18 to 20 growths, were made in 8 to 10 hours. The objections to the method have been successfully answered by Barber, Kahn, and others, and experience in this work confirms their findings, i.e., the ability to select and isolate single anaerobic cells is as well established as is any other type of laboratory technic. It is evident, of course, that single cell isolation

does not insure against later contamination, and that the same extreme care in handling such pure cultures is as necessary as in any other method of isolation.

It is particularly interesting that from four separate strains which were subjected to single cell isolation, both the A and the B types of Cl. botulinum were isolated. In each instance, the original culture produced toxin of the A type only, as demonstrated by mouse injection with specific antitoxins. The A type apparently was predominant, concealing all indications of the B type when in the mixture. Whether the fact that both A and B types were isolated from the same original stock means that both types are found growing together in such close association as to make separation impossible by the ordinary methods of isolation, or whether it means a laboratory contamination subsequent to the isolation of the stock, cannot be determined. The findings suggest the probability of both strains growing together in nature, and make it difficult to entertain the belief that different types are peculiar to certain localities.

Bengtson reports apparent mutations among Cl. botulinum, as she was able to isolate single cells from one culture, some of which developed into toxic and others into nontoxic strains. During this work, 15 to 40 cells were isolated from each strain, and all the growths obtained (2 to 12 from each culture) were tested for specific toxin production. In no instance was there any indication of the development of an atoxic strain.

CONCLUSIONS

The employment of the single cell method of isolation offers a rapid and relatively certain means of securing pure cultures of A and B types of Cl. botulinum; 800 such isolations yielded 253 growths.

The employment of this method for the isolation of single cell cultures of type C was not successful in 30 attempts.

The most suitable mediums for the cultivation of isolated single cells were found to be semisolid glucose agar, glucose broth, and pea juice gelatin.

Spore isolations yield a slightly higher percentage of growths than vegetative cells, and are best suited for isolation purposes.

The time required for isolation is not so long as to affect the practicability of the method on account of the exposure of the cells or spores to the air. No significant differences were observed in the number of growths obtained when the isolation time is kept within 10

minutes. In the case of the isolated spores, no difference was observed in the number of positive results even when the time of exposure was as much as 20 minutes.

Cl. botulinum grows and produces toxin equally well at 20 C. and 37 C. The rate of growth and of toxin production is somewhat more rapid at 37 C. The quantity of toxin eventually produced is the same at either temperature.

No atoxic strains of Cl. botulinum were obtained among the 253 single cell isolations, that were tested for toxin production.

No differences in ability to grow after exposure to air were noted between the A and B types. The same was true of the different incubation temperatures, the growth in various culture mediums; the time elapsing before growth appeared, or the degree of anaerobiosis. The A types, however, uniformly produced their toxin more rapidly, and in larger amounts than the B types.

The cultivation of single cells of Cl. botulinum in symbiosis with nonsporulating aerobes is a successful procedure, but the percentage of positive results is not enough larger to compensate for the additional labor required in inoculating with the symbiont and in freeing the mixture of the aerobes.

Isolations utilizing the culture tube method are preferable to those in which the hanging drop slide is used. They are much more easily made, yield a greater percentage of positive results, and are less likely to be contaminated.

The interval required for the appearance of growth is variable. Sixty per cent. of the positives appeared within one week. A small percentage of spore isolations exhibited prolonged periods of latency—in one case reaching 90 days. No further growths were observed after 3 months although the inoculated material was kept under observation for 6 to 8 months.

RÔLE OF OMENTUM OF RABBITS, DOGS AND GUINEA-PIGS IN ANTIBODY PRODUCTION

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It has been determined that certain organs and tissues are involved in the production of antibodies in response to intraperitoneal injection of various antigens. The main methods of attack have been the removal of organs and the inhibition or destruction of their function through the use of different physical and chemical agents, before and after the introduction of the antigenic substances.

The omentum has great phagocytic power, but it remains to determine whether it also takes part with the spleen, bone marrow, and other organs in the development of antibodies. Because of its accessibility and because it permits morphologic study of the interaction between its different cell types and intraperitoneally introduced antigens, the omentum offers a field for the examination of such relations as may exist, if any, between a local defensive reaction such as expressed by phagocytosis and a more general protective reaction evidenced by the formation of diffusible antibodies.

LITERATURE

FUNCTIONS OF THE OMENTUM

The omentum plays such a definite rôle in both normal and pathologic abdominal conditions that its importance should not be lost sight of, as a brief résumé of its various functions will clearly show.

Absorption.—That fluids are absorbed from the peritoneal cavity was known by Aristotle. The route of absorption is still in dispute. Shippley and Cunningham [1] demonstrated that "true and pseudo-solutions, and granules of particulate matter" are absorbed by the omental vessels. The lymphatic plexuses on the under surface of the diaphragm may also absorb considerable amounts of fluid and soluble elements (Bolton,[2] Buxton,[3] and Wells [4]). Ziegler [5] found that small and even relatively large foreign bodies were absorbed from the peritoneal cavity, mainly through the large network of subendothelial cells and not

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[1] Am. Jour. Physiol., 1915, 40, p. 75.
[2] Jour. Path. and Bacteriol., 1921, 24, p. 429.
[3] Jour. Med. Res., 1906, 15, p. 189; 1907, 16, p. 17.
[4] Jour. Infect. Dis., 1907, 4, p. 582; Am. Jour. Physiol., 1907, 18, p. 156.
[5] Ztschr. f. d. ges. exper. Med., 1921, 24, p. 223.

through preformed spaces. Wilkie[6] determined that more saline solution was absorbed in animals with the omentum than those deprived of this structure, in a ratio of 3 to 2. This result was confirmed by Rubin.[7] Morrison[8] likewise observed that the absorption of lymph was facilitated by increased vascularity of the omentum. Clark[9] states that the differences in the rate of absorption of various fluids from the peritoneal cavity are dependent on the variations in osmotic pressure. Posture seems to have little effect on the rapidity of absorption (Rowntree and Dandy[10]).

Reaction to Foreign Particles Introduced in the Peritoneal Cavity.—That the omentum can engulf foreign bodies introduced into the peritoneal cavity has been clearly demonstrated by Wilkie,[6] Durham,[11] Hertzler,[12] LePlay,[13] and many others. It has also been shown that animals deprived of the omentum frequently succumb to injections of charcoal. LePlay[13] found that even the resected omentum, when left in the peritoneal cavity, retained the power to react to foreign bodies.

The Movement of the Omentum to Infected Areas, and Its Plastic Effects.—It is well known that the omentum, in a considerable number of cases, seems to move to the site of acute peritoneal inflammation. There is still considerable difference in opinion as to the apparent process of motion. The omentum, because of its protective rôle, has been called "abdominal policeman," "friend in need," "the great leukocyte." Norris[14] showed that it is entirely devoid of muscle, and possesses no inherent power of movement. It seems most logical to accept the view of Rubin,[7] Fisher,[15] Bloomhardt, Andrew and Hetherington,[16] and Wilkie,[6] that in the presence of a localized peritoneal infection, active peristalsis of the intestine pushes the omentum toward the inflamed peritoneum. However, Morrison,[8] Aimes,[17] and others maintain, chiefly from clinical evidence, that the omentum changes its position with a sort of intelligence, not due primarily to mechanical causes. Morrison[8] believes that the motion is very rapid, while Wilkie[6] considers it to be a matter of hours or days before it is successfully completed.

The plastic effect has been frequently demonstrated by general surgeons, and experimentally by Bloomhardt, Andrews and Hetherington.[16] These authors found that the omentum was capable of closing experimental perforations. Many older surgeons—Senn, Mayo, and others—have shown the great clinical value of using the omentum to repair defects in the gastro-intestinal tract, and sealing tight the line of suture in intestinal anastomosis.

The Bactericidal Function of the Omentum.—The omentum contains many phagocytic cells (Metchnikoff), singly and in islands along the blood vessels, and Wilkie[6] secured positive bacterial cultures from the omentum, when the exudate had become sterile after injection of broth cultures into the peritoneal cavity. Norris[14] believes that the main function of the omentum is that of leukocytogenesis. Buchtel[18] found that animals deprived of the omentum were less resistant

[6] Brit. Med. Jour., 1911, 2, p. 1103.
[7] Surg., Gynec. & Obst., 1911, 12, p. 117.
[8] Brit. Med. Jour., 1906, 1, p. 76.
[9] Jour. Pharm. & Exper. Therap., 1921, 16, p. 415.
[10] Ann. Surg., 1914, 59, p. 44.
[11] Jour. Path. & Bacteriol., 1892, 4, p. 338.
[12] The Peritoneum, 1919.
[13] Compt. rend. Soc. de biol., 1911, 71, p. 484.
[14] Univ. Pennsylvania Med. Bull., 1908, 21, p. 119.
[15] Brit. Med. Jour., 1906, 2, p. 325.
[16] Surg., Gynec. & Obst., 1917, 34, p. 474.
[17] Progrès méd., 1920, 35, p. 8, 12.
[18] Denver Med. Times and Utah Med. Jour., 1911, 31, p. 305.

than normal animals to induced peritoneal infection. Dudgeon and Ross [19] have demonstrated that the omentum becomes intensely injected in experimental peritonitis, and also confirmed the findings of Wilkie. Rubin [7] likewise agrees with the other authors in the great phagocytic power of the omentum.

Vascularization of Tumors and Reaction to Strangulated Organs.—De Renzi and Boeri [20] have found that when the blood supply of the spleen or kidney was ligatured, the omentum usually encapsulated the infarcted organ and the animals survived, whereas when the omentum was previously removed, they invariably died. Furthermore, they determined that the omentum could supplement a partial interference with the arterial supply of the spleen. The spleen, in the animals which survived the complete occlusion of vascular supply, was completely encapsulated in the omentum. This function has been further studied in experimental strangulation of large segments of the gastro-intestinal tract (Bloomhardt, Andrews and Hetherington [16] and others). The vascularization of abdominal tumors through omental adhesions has been seen by all surgeons and pathologists. The readiness with which a rich vascular supply may be developed through omental adhesions is utilized in the various operations of omentopexy for cirrhosis of the liver.

Summary.—The omentum is an important factor in absorption; it is capable of walling off localized peritoneal infections, and of encapsulating foreign bodies; it has considerable anti-infectious power; and it may be used to furnish a new vascular supply to strangulated organs.

THE STRUCTURE OF THE OMENTUM

The omentum of many different species has been studied carefully. The rabbit omentum, however, has been the one of choice for experimental work. Norris,[14] in a complete review of this structure, described it accurately:

The omentum consists of a serous membrane, which hangs, apron like, from its attachments to the stomach, transverse colon, gastrohepatic and gastrosplenic ligaments. It consists of 4 layers of peritoneum between which are enclosed arteries, veins, lymphatics, nerves, fat, and fibrous tissue, also many cellular accumulations. The arterial supply is derived from the gastro-epiploic, middle colic, splenic, and duodenal arteries. These vessels send off numerous treelike branches, usually at right angles to the main trunks. There are, however, many avascular areas. The cellular aggregations are chiefly found along the vessels. The lymphatics form a well defined system, the larger vessels of which follow the larger arteries, and have the usual structure, consisting of an intima, media and adventitia. The smaller lymphatics consist of a single layer of endothelium. Norris believes that there are definite stomata, but later work seems to have disproved this. Hertzler [12] states that the lymphatics are poorly defined and few in number.

Cunningham [21] considered the rabbit omentum a characteristic structure, consisting of a framework of blood vessels which anastomose freely without the

[19] Am. Jour. Med. Sc., 1906, 132, p. 37.
[20] Berl. klin. Wchnschr., 1903, 34, p. 773.
[21] Bull. Johns Hopkins Hosp., 1922, 33, p. 257.

intervention of an elaborate capillary bed and that much of the circulation is arteriovenous in character. The larger vessels are surrounded by layers of adipose tissue. The thin membranous part consists of numerous cells interspersed in a mass of interlacing connective tissue fibrils and covered by a layer of flattened mesothelial cells, whose outlines can be demonstrated by the mosaic pattern which they evidence after treatment with silver nitrate. The special structures which are so evident in the rabbit omentum are the "taches laiteuses" scattered more or less indiscriminately throughout the omentum. These "taches laiteuses" or milky spots are formed by collections of various types of free cells grouped about areas of increased vascularity. These cellular islands were first described by Ranvier, so that they are frequently called "taches laiteuses de Ranvier." Weidenreich [22] believed that the omentum was wholly analogous to a lymph gland, the mesh work corresponding to the reticulum of the gland and the free cells and the "taches laiteuses" to the pulp cells and the follicles, respectively.

The histology of the omentum has afforded an interesting field for research, because of its varied cellular content. Cunningham [21] has recently stated that the work along this line could be divided into two great groups: one including the genetic classification of the cells which constitute the "taches laiteuses" of the rabbit omentum; the other concerning the characteristics and relationship between the three great groups of cells which constitute the framework of the organ—the clasmatocytes, fibroblasts, and the cells of the serosal lining.

The relation of the serosal cells to the fibroblasts has aroused considerable discussion. Ranvier,[23] in 1891, noticed that after setting up a peritoneal irritation with silver nitrate the superficial cells were shed and seemed to be replaced by the deeper cells, and hence concluded that the two types of cells were indistinguishable and interchangeable. Dominici,[24] in 1901, considered the component cells of the omentum as representing various cell syncytiums which were not entirely separated but belonged collectively to the connective tissue series and were interchangeable. Schott [25] came to the same conclusion after his minute study of the inflammatory processes involving the omentum. However, Kiyono, quoted by Cunningham, in examining omental spreads from animals vitally stained with carmine and trypan blue, could never find any indication of a transition from the lining cells to fibroblasts. He said that normally the lining cells form a continuous and characteristic cell series over the omental surfaces, and they can be easily differentiated from the fibroblasts; but in the presence of inflammation the two types may resemble each other closely. Von Büngner [26] came to a similar conclusion in work on granulation tissue production, as he could not detect any transition between the two groups of cells. Tschasin [27] later decided that he could differentiate definitely between the serosal cells and the fibroblasts by means of the vital dyes, as the former type failed to take on the stains. Cunningham [21] noted that the fibroblasts took on more vital dyes than the serosal cells, but neither type reacted intensively. In his preparations of normal omentums which were vitally stained and silvered, the fibroblasts were seen in between the two layers of serosal cells as cells with faint outlines and finely granular inclusions in the cytoplasm. These fibroblasts were often branched, and

[22] Arch. f. mikr. Anat., 1908, 72, p. 209.
[23] Compt. rend. Acad. d. sc., 1891, 112, p. 842.
[24] Compt. rend. Soc. de biol., 1901, 53, p. 890.
[25] Arch. f. mikr. Anat., 1909, 74, p. 143.
[26] Beitr. z. path. Anat. u. z. allg. Path., 1896, 19, p. 33.
[27] Folia haemat., 1913, 17, p. 317.

even though the outlines were indistinct, they could be made out because of the trypan blue in the cellular processes. In a later paper [28] on the reactions of the cells of the peritoneal cavity to vital dyes, he showed that the large serosal lining cells stained characteristically, as most of them contained a small ring of granules in one part of the cytoplasm. This method of storage of vital dye was considered characteristic, especially the localization of the granules in a circumscribed area in the cytoplasm and in the formation of a perinuclear rosette. It appears that the serosal cells and the fibroblasts are distinct cell types, although they appear to be closely related when subjected to external influences.

The origin of the clasmatocytes has also aroused considerable interest. Ranvier,[29] writing in 1899 on "Les clasmatocytes," stated that he first noticed the presence of the clasmatocytes in the femoral aponeurosis. Later he used the mesentery of the molluscs, in which he found masses of cells which resembled the ordinary leukocytes. These clasmatocytes had dendritic processes, and he considered them as derived from the fibroblasts. His further work with the dorsal lymph space of the frog and the rabbit omentum led him to conclude that the clasmatocytes were distinct from the fibroblasts. He then concluded that the clasmatocytes were derived from the leukocytes. The separation of the clasmatocyte as a distinct type of cell of the connective tissues was due to the work of Maximow.[30] He showed that by the introduction of two sterile cover slips under the skin of rabbits, one could separate 3 types of cells by the speed with which they passed between the covers: the leukocytes appearing first; then a special cell, the clasmatocyte, which wandered in in about 19 hours, and lastly the fibroblasts. He further showed that the clasmatocytes were specifically sensitive to neutral red. Later workers have confirmed these results and have concluded that the clasmatocyte is a cell of connective tissue origin, specifically differentiated to take up and store particulate matter. Sabin,[31] from her careful work on the chick, concluded that the endothelium gives rise to 2 groups of cells: the megaloblasts which develop hemoglobin and become erythroblasts, and a strain of cells called histiocytes by Aschoff. The extravascular histiocytes have been termed clasmatocytes; the intravascular cells are called monocytes. She stated that the latter group is specifically differentiated for purposes of phagocytosis. Dominici [32] considered the macrophages as arising from the differentiations of the various cell syncytiums which are normally present in the omentum, probably from the primitive cells. Foot [33] reached a conclusion similar to Sabin's.

Cunningham,[21] in his omental preparations, described an additional series of cells which were elongated and loaded with trypan blue. Some of these cells were oval and found chiefly in the "taches laiteuses." He considered these typical clasmatocytes, similar to the "adventitial" cell of Marchand. Many of the cells formed in long lines along the course of the vessels, and were at times intimately connected with the endothelial cells of the capillaries. Dominici also considered the macrophages as mainly limited to the "taches laiteuses." Weidenreich [22] limited the anlage of exudative cells of the peritoneal cavity to the omentum, and later Cunningham believed the clasmatocytes contained in the peritoneal exudate of the rabbit to be derived from the cellular constituents of the "taches laiteuses."

[28] Am. Jour. Anat., 1922, 30, p. 399.
[29] Arch. d'anat. micr., 1899, 3, p. 122.
[30] Beitr. z. path. Anat. u. z. allg. Path., 1901, 4, p. 1; Arch. f. mikr. Anat., 1906, 67, p. 680
[31] Bull. Johns Hopkins Hosp., 1921, 32, p. 314.
[32] Arch. de méd. expér. et d'anat. path., 1902, 14, p. 1.
[33] Jour. Med. Research, 1919, 40, p. 353.

THE SITES OF ANTIBODY PRODUCTION

Ehrlich's lateral chain theory assumes that any cell that takes and binds antigen can produce the corresponding antibody or antibodies. Hektoen [34] states that whether the power to produce antibodies is widely distributed among cells of the body or whether it is limited more or less strictly to certain cells or tissues has not been definitely determined. Considerable effort has been spent in the attempt to determine the organs and tissues involved in the production of antibodies.

In general, the methods used have been the removal of organs which were considered as possible sites of antibody production; the use of various physical and chemical agents, generally chosen so as to have some selective action on a special tissue, and the use of different sites for the introduction of the antigens.

The hematopoietic system has been considered the most important integral part of the mechanism for the fabrication of antibodies. Certain experiments have been made to establish this assumption. Chief have been those on the effects of splenectomy and alterations in the bone marrow and lymphatic apparatus by physical and chemical agents on the subsequent course of antibody production. Hektoen [35] showed that splenectomy may diminish the output of antibodies, especially when practiced about the same time the antigen is introduced, but had little or no effect when the antibody production was well under way. Motohasi [36] came to similar conclusions, in that splenectomy in rabbits profoundly modified the production of specific hemolysin, both as to site and concentration in the various organs and in the blood stream. He also noted a compensatory hemophage activity, in splenectomized rabbits, in the bone marrow and liver after about 45 days. Hektoen had previously noted this compensatory property of the tissues concerned in antibody activity. Krumbhaar and Musser [37] found that splenectomized monkeys seemed to exhibit much less blood change than splenectomized dogs or men. The removal of the stomach, small intestines, and thyroid gland was found by Hektoen and Curtis [38] to have no effect on antibody production.

Various chemical and physical agents have been used to bring about reactions in the hematopoietic system. Thompson [39] was able to increase the antibody producing power of rabbits by preliminary injections of tuberculin; this reaction he attributed to the irritating effects on the endothelial phagocytes. Arkin [40] demonstrated that strychnine had a stimulating effect, while morphine, potassium cyanid and chloral had a depressing effect on phagocytosis. Sodium iodoxybenzoate has been found to stimulate lysin production in dogs, an effect which Hektoen [41] and Arkin [42] considered as probably due to acceleration of oxidation in the tissues involved in antibody production. Hektoen [43] also demonstrated that repeated injections of benzene in rabbits at the same time that sheep blood was injected greatly reduced the concentration of specific precipitins and lysins. The same author and Corper [44] noted that such agents as thorium X and mustard

[34] Jour. Infect. Dis., 1911, 9, p. 103.
[35] Ibid., 1920, 27, p. 23.
[36] Jour. Med. Res., 1922, 43, p. 419, 473.
[37] Arch. Int. Med., 1923, 31, p. 686.
[38] Jour. Infect. Dis., 1915, 17, p. 409.
[39] Jour. Med. Res., 1922, 43, p. 37.
[40] Jour. Infect. Dis., 1913, 13, p. 408.
[41] Tr. Chicago Path. Soc., 1911, 8, p. 138.
[42] Jour. Infect. Dis., 1915, 16, p. 349.
[43] Ibid., 1916, 19, p. 69.
[44] Ibid., 1920, 26 p. 320; 1921, 28, p. 279.

gas had a similar restraining effect on antibody elaboration. In the experiments of the latter type the depressant action of the chemical agents on antibody formation is associated with a marked decrease in the circulating leukocytes and with destruction of the leukocyte precursors in the bone marrow.

The roentgen ray and radioactive substances have profound effects on the lymphatic and blood forming apparatus. Nakahara [45] has shown that there is a marked stimulation of the lymphoid tissue subsequent to small doses of radiation. Murphy and Morton [46] had similar results. Russ and his co-workers [47] found that large doses of radiation caused a marked decrease in the circulating lymphocytes. Hektoen [48] demonstrated that roentgen-ray exposure of rats before immunization reduced the lysin formation, whereas this agent had no definite effect after the process was well under way. He had similar results with the simple exposure of dogs and also when splenectomy was combined with the radiation. Likewise, radium emanation given intravenously in sublethal doses had a depressing effect on lysin production (Hektoen and Corper [49]). Ivy and his co-workers [50] do not believe that the effects of radiation on the spleen and lymph glands are due to stimulation but rather to irritation.

Zinsser [51] stated that the tissue cell, as the functional unit, must be looked on as the source of the various protective constituents of normal and immune serum. Kyes [52] and later Cary [53] found that the organs with great hemophage activity, in the destruction of foreign red blood corpuscles, have a higher concentration of specific antibody. Kyes postulated the theory that the hemophages are the producers of antibodies specific for cells they take up. Cantacuzene,[54] in trying to locate the formation of precipitins, found that the spleen, bone marrow and lymph nodes contained precipitins in increasing amount up to the seventh day of immunization and on that day the precipitins appeared in the blood. Also that extracts of leukocytes, obtained in the pleural exudates resulting from aleuronat injections, contained precipitins earlier than the blood. Since mononuclear cells predominate in the experimental exudate, and precipitins are found first and in the highest concentration in the organs rich in mononuclears, he concluded that these cells probably form the precipitins. Krause and Schiffman [55] were unable to demonstrate the presence of precipitins in the extracts of various organs of the rabbits although the serum contained this antibody.

Many writers have attempted to prove that tissues, other than those forming the hematopoietic system, may be included in the antibody formers. Römer [56] found that abrin injected into the conjunctival sac caused the local formation of antitoxin at a stage corresponding to antitoxin production called forth by abrin, which is absorbed into the general circulation. Hektoen [35] after injection of antigen into the anterior chamber of dogs was unable to find any evidence of antibody in the aqueous humor until after newly formed antibodies appeared in

[45] Jour. Exper. Med., 1919, 24, p. 83.
[46] Ibid., 1915, 22, p. 800.
[47] Lancet, 1919, 1, p. 692.
[48] Jour. Infect. Dis., 1915, 17, p. 415; 1918, 22, p. 28; 1920, 27, p. 23.
[49] Ibid., 1922, 31, p. 304.
[50] Jour. Radiol., 1923, 4, p. 189.
[51] Infection and Resistance, 1918, p. 200.
[52] Jour. Infect. Dis., 1916, 18, p. 277.
[53] Jour. Med. Res., 1922, 43, p. 399.
[54] Ann. de l'Inst. Pasteur, 1908, 22, p. 54.
[55] Ibid., 1906, 20, p. 225.
[56] Arch. f. Ophth., 1901, 52, p. 72.

the blood. Wassermann and Citron[57] injected typhoid bacilli intravenously, intraperitoneally, and intrapleurally into dogs. They then injected 5 c c. of aleuronat on the 9th day into the pleural cavity and 10 c c. of normal salt solution intraperitoneally. The dogs were bled the next day and the pleural and peritoneal exudates withdrawn. Their results indicated the possibility of the local formation of antibody by the cells of the peritoneum and pleura. Hektoen,[34] however, found that in most cases intrapleural injection of the antigen gave less concentration of antibody in the serum than intravenous injection, and in no case after intrapleural injection of the antigen did the antibody content in the pleural exudate exceed that of the serum. Klauder and Kolmer[58] found that the local Wassermann reaction was of much more value than the blood test in primary syphilis, and that it yielded as high a percentage of positive reactions as the dark field method. Russell[59] claimed that the subcutaneous injection of typhoid vaccine in typhoid fever is beneficial. He attempted to explain his results by the inherent power of the connective tissue to form antibodies, and supposed that this type of tissue in the subcutaneous region does not normally react in the usual course of the disease. Hektoen[34] was unable to confirm the increased production of antibodies after multiple subcutaneous injections of the antigen. The recent work of Gay and Morrison[60] has shown that successful resistance to streptococcus pleurisy is constantly associated with a persistently high clasmatocyte count. They consider that this local form of immunity to streptococcus is associated with the "tissue macrophages" or clasmatocytes.

The experimental work reviewed points to a widespread distribution in the body of the function of forming soluble antibodies. But it has not succeeded in proving that antibody formation is a specific property of any particular tissue or cell type. It indicates that those groups of tissues and cell species which make up the hematopoietic system are most active, but it has not as yet definitely determined which of the cell constituents of this system are chiefly concerned in the elaboration of antibodies; nor has it shown to what degree this function may reside in certain cell types, situated outside the hematopoietic tissues, which are common to the latter and the other tissues of the body.

EXPERIMENTS

Rabbits, guinea-pigs and dogs were used. The operative procedures were carried out under ether anesthesia. The omentum was removed after careful ligation of the larger arteries and veins as they leave the gastroepiploic vessels along the greater curvature of the stomach. Care was taken not to interfere with the splenic artery as it passes through the upper border of the gastrosplenic omentum. The animals were

[57] Ztschr. f. Hyg. u. Infektionskrankh., 1905, 50, p. 331.
[58] Jour. Am. Med. Assn., 1921, 76, p. 1635.
[59] Boston Med. & Surg. Jour., 1911, 164, p. 1.
[60] Jour. Am. Med. Assn., 1923, 80, p. 1298; Jour. Infect. Dis., 1923, 33, p. 338.

allowed to recuperate for a period of two weeks, after which time the antigen was introduced into the peritoneal cavity through a small incision in the abdominal wall. They were then bled every 2 days. Healthy normal animals were used as controls.

TABLE 1

INTRAPERITONEAL IMMUNIZATION OF RABBITS WITH DEFIBRINATED SHEEP BLOOD

Days	Rabbit 2			Rabbit 4			Control Rabbit 1		
	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins
2	24	10	0	12	10	0	6	160	0
5	96	80	0	384	40	12	1,536	640	96
7	384	320	24	1,536	640	96	3,072	2,560	192
9	768	640	48	3,072	2,560	192	12,288	5,120	768
12	3,072	1,280	96	6,144	2,560	384	24,576	5,120	1,536
14	6,144	1,280	96	6,144	2,560	192	24,576	5,120	768
16	3,072	640	96	6,144	2,560	96	12,288	2,560	384
19	1,536	640	96	3,072	1,280	48	6,144	1,280	192
21	1,536	320	48	3,072	1,280	48	6,144	1,280	96
23	1,536	320	24	1,536	1,280	48	3,072	1,280	48
25	1,536	320	24	768	1,280	24	3,072	1,280	96
28	768	160	24	768	640	24	3,072	640	96
30	384	160	24	384	640	24	1,536	640	48
34	384	80	24	384	320	12	768	320	24
37	192	40	12	384	320	6	768	320	24
41	192	20	12	192	80	6	384	80	24
44	192	20	6	192	80	6	384	80	12
50	96	10	6	96	40	6	192	80	12
60	96	10	3	96	20	6	192	40	6
79	0	0	0	48	10	0	96	10	6

TABLE 2

INTRAPERITONEAL IMMUNIZATION OF DOGS WITH DEFIBRINATED SHEEP BLOOD

Days	Dog 2		Dog 3		Control Dog 1		Control Dog 2	
	Lysins	Agglutinins	Lysins	Agglutinins	Lysins	Agglutinins	Lysins	Agglutinins
1	0	0	0	0	0	0	0	0
3	12	12	12	6	12	12	24	6
5	768	48	384	48	768	96	3,072	48
7	6,144	192	3,072	96	6,144	384	6,144	192
9	24,576	384	12,288	192	24,576	768	12,288	384
12	12,288	192	6,144	96	12,288	384	12,288	192
14	12,288	192	6,144	96	6,144	192	6,144	96
16	6,144	96	3,072	48	3,072	96	3,072	48
18	3,072	96	1,536	48	1,536	96	3,072	48
20	1,536	96	768	48	768	48	3,072	24
23	768	48	768	24	192	48	1,536	24
24	384	48	384	12	96	24	768	12
28	192	24	192	6	96	12	192	6
30	96	12	48	6	48	6	96	6
40	96	6	24	0	48	6	96	6
50	48	6	12	0	48	0	48	0
60	24	0	6	0	24	0	48	0
70	24	0	6	0	12	0	24	0

The serum was separated from the blood, heated at 56 C. for 30 minutes, and then the antibody concentration determined. For lysin content, mixtures were made of 0.2 c c. of a 5% suspension of washed sheep or chicken corpuscles in normal salt solution; 0.003 c c. of guinea-

pig serum as complement (0.006 c c. was necessary for the chicken hemolytic system); and dilutions of the rabbit, dog or guinea-pig serum to be tested in normal salt solution. The total volume in each case was 0.6 c c. This mixture was incubated at 37 C. for 2 hours and then placed in the icebox over night. Next morning the highest dilution of the serum causing hemolysis was recorded. For the precipitin concentration, the highest dilution of sheep or chicken serum in which the rabbit, dog or guinea-pig serum caused a definite precipitate, by the contact method, after standing at room temperature for 1 hour, was recorded. The agglutinin titers were determined by means of mixtures of 0.2 c c. of a 5% suspension of washed sheep or chicken corpuscles and dilutions of inactivated rabbit, dog or guinea-pig serum, in salt solution, the total volume in each case being 0.6 c c. These mixtures were incubated for 2 hours and then placed in the icebox over night, and next morning the highest dilution of each serum causing distinct agglutination of the corpuscles was noted.

Removal of the Omentum and Intraperitoneal Immunization.—The omentum was removed from a series of rabbits as described. Two weeks later, under light anesthesia, a small incision was made through the anterior abdominal wall and 15 c c. of defibrinated sheep blood were introduced. Several controls were immunized in a similar manner at the same time. The rabbits were bled from the marginal ear vein every 2 days, and the concentration of the antibodies in the serum determined by the methods indicated above. The results are given in table 1 and chart 1. The experiments were repeated several times with similar results.

Two weeks after removing the omentum in a number of dogs, 25 c c. of defibrinated sheep blood were introduced intraperitoneally. Several controls were immunized at the same time in a similar manner. The dogs were bled every two days and the concentration of the antibodies determined (table 2 and chart 2).

Similar experiments were made in guinea-pigs, using 5 c c. of defibrinated sheep blood. These animals were bled from the heart at frequent intervals and the antibody content determined (table 3).

As seen in tables 1-3, and charts 1 and 2, the general course of the antibody curves corresponds to that of the typical curve following a single injection of antigen described by Hektoen and Carlson.[61] The control rabbits produced about 4 times the quantity of antibodies at the height of the curve as did the rabbits deprived of the omentum, and

[61] Jour. Infect. Dis., 1910, 7, p. 319.

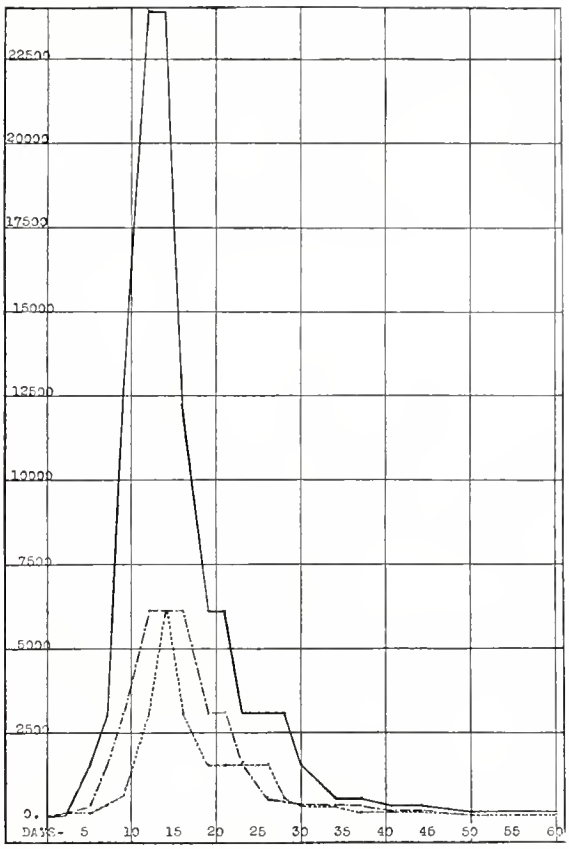

Fig. 1.—Lysins produced in rabbits following intraperitoneal injection of sheep blood The dash and dot line indicates the omentum removed; the dotted line, the omentum removed; the continuous line, the omentum intact.

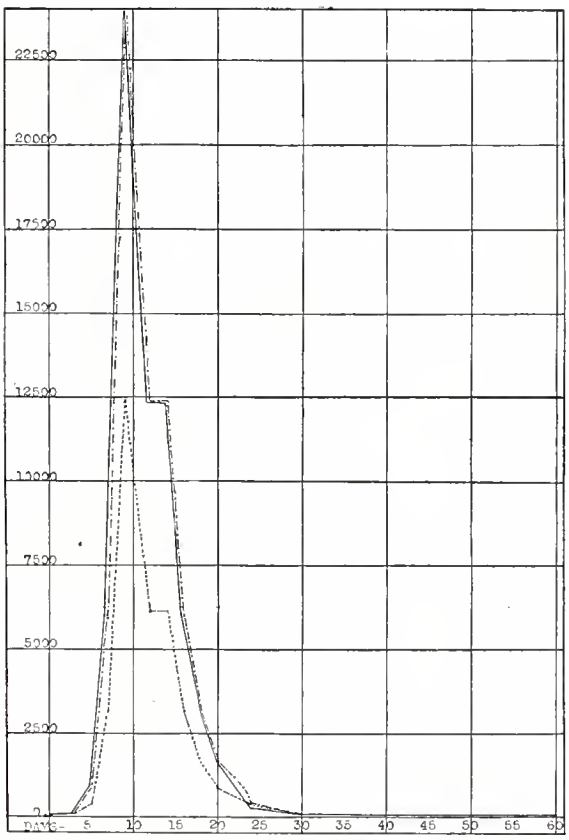

Fig. 2.—Lysins produced in dogs following intraperitoneal injection of sheep blood. The dash and dot line indicates the omentum removed; the dotted line, the omentum removed; the continuous line, the omentum intact.

TABLE 3

INTRAPERITONEAL IMMUNIZATION OF GUINEA-PIGS WITH DEFIBRINATED SHEEP BLOOD

Days	Guinea-Pig 3			Control Pig 3			Control Pig 4		
	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins
3	0	0	0	0	0	0	0	0	0
6	0	0	6	0	0	6	0	0	6
9	6	0	12	0	0	12	6	0	12
12	12	0	24	6	0	12	12	0	24
14	96	0	48	12	0	12	96	0	48
16	48	0	24	6	0	12	48	0	24
18	24	0	24	0	0	6	24	0	12
21	6	0	12	0	0	0	12	0	6

TABLE 4

INTRAVENOUS IMMUNIZATION OF RABBITS WITH DEFIBRINATED SHEEP BLOOD

Days	Rabbit 19			Rabbit 20			Control Rabbit 20		
	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins
1	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	24	10	6
5	96	20	6	96	20	6	384	160	24
7	768	160	48	768	160	48	1,536	320	48
9	1,536	640	96	1,536	320	96	1,536	640	96
11	1,536	1,280	96	1,536	640	96	1,536	1,280	96
13	768	640	48	768	320	48	1,536	640	48
15	768	640	24	384	160	24	768	320	48
17	384	320	12	384	160	12	768	320	24
19	192	160	6	192	80	6	384	160	12
21	96	80	6	96	40	0	192	80	12
23	96	40	0	48	40	0	96	80	6
25	48	10	0	24	10	0	96	40	6
27	24	10	0	12	0	0	48	10	6
30	12	10	0	6	0	0	24	10	0

TABLE 5

INTRAVENOUS IMMUNIZATION OF DOGS WITH DEFIBRINATED SHEEP BLOOD

Days	Dog 4		Dog 5		Control Dog 3	
	Lysins	Agglutinins	Lysins	Agglutinins	Lysins	Agglutinins
2	0	0	0	0	0	0
4	6	0	6	6	6	0
6	192	48	192	48	96	24
9	384	48	384	96	192	48
11	768	96	768	96	768	96
13	1,536	192	1,536	192	1,536	192
16	768	96	1,536	192	768	96
18	768	48	768	96	384	48
20	384	24	384	48	192	24
23	192	12	192	48	96	12
25	192	6	96	24	96	6
27	96	6	48	12	48	6
30	48	6	24	6	12	6

the active antibody production continued longer in the control rabbits. In dogs and guinea-pigs, however, there was no marked difference in the concentration of antibodies produced by the animals with and without the omentum.

Removal of the Omentum and Intravenous Immunization.—Two weeks after removing the omentum from a number of rabbits and dogs, 2 c c. of defibrinated sheep blood were injected into the ear vein of the rabbits and the external jugular vein of the dogs. At the same time control animals were injected in the same way. The animals were subsequently bled every 2 days and the antibody content of the serum determined. The results in the rabbits are recorded in table 4 and those of the dogs in table 5.

TABLE 6

INTRAPERITONEAL IMMUNIZATION OF RABBITS WITH DEFIBRINATED SHEEP BLOOD AND REMOVAL OF THE OMENTUM DURING THE COURSE OF ANTIBODY PRODUCTION

Days	Rabbit 21			Rabbit 22			Rabbit 23		
	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins
Normal	0	0	0	0	0	0	0	0	0
2	96	0	6	96	0	6	192	0	6
4	192	10	12	192	10	12	384	10	12
4		Omentum removed							
8	384	20	24	768	80	48	1,536	40	96
8					Omentum removed				
10	1,536	320	48	768	160	48	3,072	640	192
12	3,072	640	96	3,072	640	96	6,144	1,280	192
12								Omentum removed	
14	3,072	640	96	6,144	1,280	192	12,288	2,560	384
17	.....	.....	...	3,072	640	96	6,144	640	192
20	.....	.....	...	1,536	320	48	3,072	320	96

As seen in tables 4 and 5, the rabbits deprived of the omentum produced about an equal concentration of antibodies in the serum as the control animals, after intravenous injection of the antigen. The dogs also reacted in a similar manner.

Sheep serum was then used as a soluble antigen and the previous experimental work repeated. Similar results were obtained as with sheep blood. Chicken blood was substituted for the sheep blood, and like results were noted.

Removal of the Omentum at Varying Stages of Antibody Production.—In another series of rabbits, 5 c c. of defibrinated sheep blood were injected intraperitoneally, and the omentum was removed on the 4th, 8th and 12th days. They were bled every 2 days, and the concentration of antibodies in the serum was determined (table 6).

Removal of the omentum during the process of antibody production had a definite influence on the subsequent antibody course. This was most marked when the omentum was removed at the end of 4 days, and less marked after 8 days.

Removal of the Omentum and Spleen with Intraperitoneal Immunization.—In other rabbits and dogs, 2 weeks after removing the omentum, the spleen was removed and defibrinated sheep blood introduced into the peritoneal cavity, 15 c c. in each rabbit, and 25 c c. in each of the dogs. At the same time, animals from which only the omentum had been removed, as well as suitable controls, were immunized in a similar manner. These animals were bled at frequent intervals and the antibody content determined (table 7).

TABLE 7

REMOVAL OF THE OMENTUM AND SPLEEN IN RABBITS WITH INTRAPERITONEAL IMMUNIZATION WITH DEFIBRINATED SHEEP BLOOD

Days	Rabbit 12, Spleen and Omentum Removed			Control Rabbit 10 Omentum Removed			Control Rabbit 11 No Operations		
	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins
2	48	20	12	48	10	6	96	20	12
4	96	40	24	192	80	24	384	160	48
6	768	320	48	768	320	48	1,536	640	96
7	1,536	640	192	3,072	640	384	6,144	2,560	768
9	3,072	640	384	6,144	1,280	768	12,288	2,560	768
11	6,144	1,280	768	12,288	2,560	1,536	24,576	5,120	1,536
13	6,144	1,280	768	6,144	2,560	768	24,576	5,120	1,536
15	3,072	1,280	384	6,144	1,280	384	12,288	2,560	768
16	1,536	640	192	3,072	640	192	6,144	1,280	384
18	1,536	640	192	3,072	640	192	3,072	1,280	384
21	768	320	96	1,536	320	96	1,536	640	192
23	384	320	48	768	320	96	768	640	96
25	384	320	48	768	320	48	768	640	96
30	384	160	48	768	160	48	768	320	96

It was found, first, that splenectomy alone produced a definite decrease in antibody production. When splenectomy followed the removal of the omentum there was a more marked decrease than after simple removal of the omentum.

Omental Transplants and Their Influence on Antibody Production.—The omentum was removed from several rabbits and dogs and, in 2 weeks omentum from normal animals was transplanted into the peritoneal cavity of the experimental animals. In a few, the omentum was transplanted in one piece spread over the abdominal viscera. In others, the omentum was cut into small pieces of about 1 sq. cm., and inserted in various places. The animals were killed at the end of two weeks,

and the omental transplants studied histologically. It was found that with both methods of transplantation the omentum soon became converted into acellular fibrous masses which were adherent to the peritoneum.

In similar series, defibrinated sheep blood was injected at the time of omental transplantation, and the subsequent course of antibodies noted (table 8).

TABLE 8

Transplanted Omentum and Intraperitoneal Immunization of Rabbits with Defibrinated Sheep Blood

Days	Rabbit 17 Transplanted Omentum			Rabbit 18 No Omentum			Control Rabbit 19 Normal		
	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins	Lysins	Precipitin	Agglutinins
1	0	0	0	0	0	0	12	0	0
2	0	0	0	6	0	0	48	10	6
4	6	0	6	24	10	6	192	20	12
6	192	80	12	384	160	12	768	320	48
8	768	320	24	1,536	640	48	3,072	1,280	96
10	1,536	640	24	3,072	640	48	6,144	2,560	192
12	3,072	1,280	96	6,144	1,280	196	12,288	5,120	768
14	3,072	1,280	48	6,144	1,280	192	24,576	5,120	768
16	1,536	640	24	3,072	1,280	96	12,288	2,560	384
18	1,536	640	24	1,536	640	48	6,144	1,280	192
20	768	320	12	768	320	24	3,072	640	96
22	768	160	12	384	160	12	1,536	320	96
24	768	160	12	384	160	12	1,536	320	96
26	384	80	6	192	80	12	1,536	320	96
28	384	80	6	192	80	6	1,536	320	48
30	192	40	6	192	80	6	768	160	24

As indicated in table 8, rabbits with transplanted omentum did not produce more antibodies than rabbits deprived of the omentum, both giving less than the normal controls.

MORPHOLOGY OF THE OMENTUM

The excised omentum was fixed in a 3% solution of potassium bichromate in 10% formalin for 24 hours and then washed in distilled water for 12 hours. Portions of the thinner parts were used for permanent preparations. Two methods of staining were found most suitable:

1. The usual hematoxylin-eosin stain.

2. Grenacher's alum carmine method, which consisted in staining the tissue 24 hours in 2% carmine in a 5% potassium alum solution. The tissue was then washed in distilled water until it was free from the excess of stain and was then immersed in a saturated alcoholic picric acid solution for 5 minutes, dehydrated, cleared in creosote and finally

mounted as a stretched preparation in balsam. The latter method stained the nuclei red, so that it could be used as a good counterstain for vital staining with trypan blue.

Rabbit Omentum.—The omenta removed from all the experimental rabbits showed moderate variations in gross and microscopic appearances. There were, however, marked differences in size according to the age. The young rabbits had a thin, small, membranous structure, whereas the mature animals had a well defined and in some cases extensive membrane, which frequently measured 13 by 7 cm. The tissue was

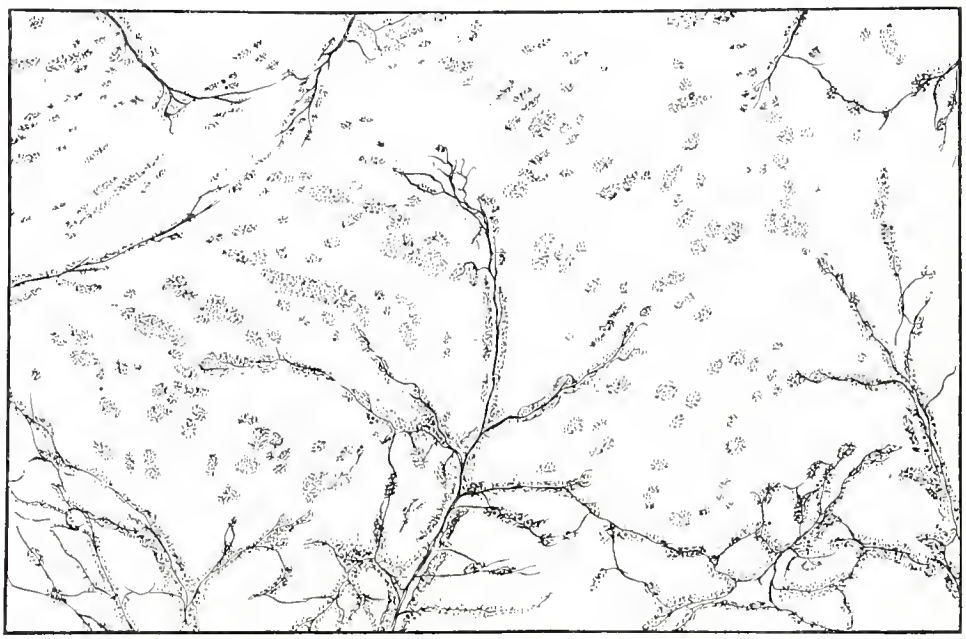

Fig. 3.—Sketch of the rabbit omentum showing the characteristic gross appearance of this structure with the branching blood vessels and numerous "taches laiteuses."

transparent except at the insertion along the greater curvature of the stomach, and the splenic and duodenal regions, where it was somewhat fibrous and contained adipose tissue. The vessels formed a treelike structure, with small cellular areas scattered along them. These "taches laiteuses" were equally numerous throughout the membrane, and many had no demonstrable vascular connections.

Specimens, fixed and stained according to the methods given, revealed a uniform and peculiar structure (fig. 3). There were two definite mesothelial layers, between which were the fibroblasts, clasmatocytes, and vessels. The "taches laiteuses" had a characteristic struc-

ture (fig. 4). Those that occurred along the blood vessels had the appearance of a glomerulus, formed by interlacing capillary tufts with well developed afferent and efferent vessels; however, there were many cellular islands which have no demonstrable vascular connections. In addition to the vascular structures of the large islands, there were many cells scattered through them, some of which were elongated and oval, while others were more round and had smaller nuclei. Many of the

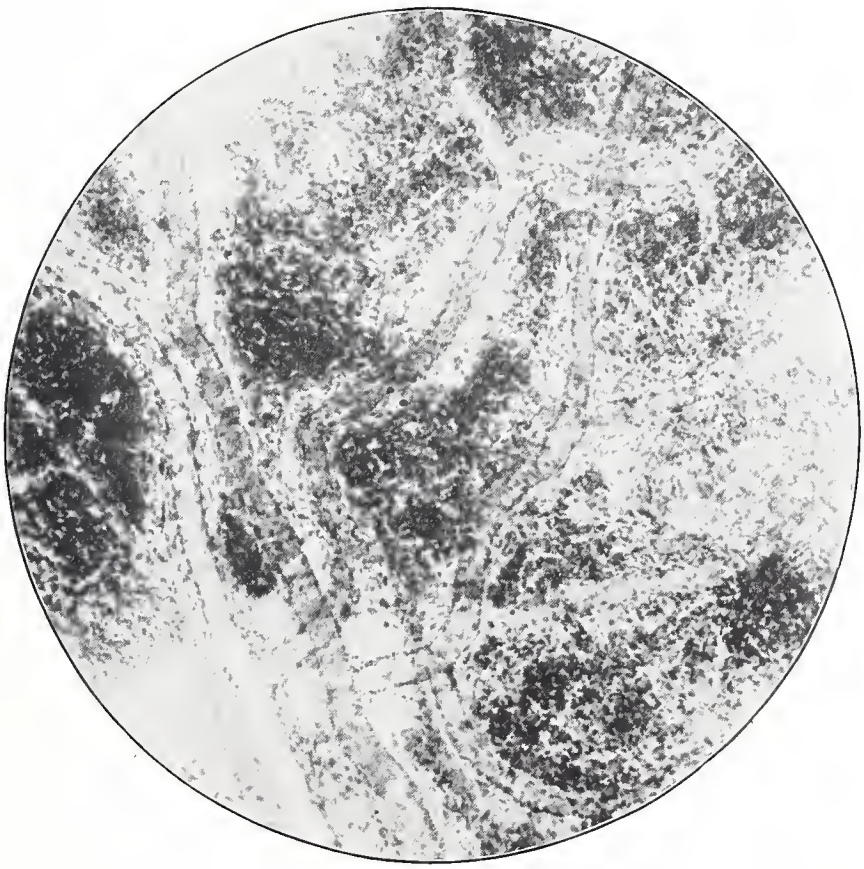

Fig. 4.—Low power representation of a vascular "tache laiteuse" of a rabbit omentum, showing the interlacing capillary tufts and extensive cellular accumulations. × 85.

cells had long branching intercellular processes. These different cell types were brought out clearly by the use of vital stains, to be described.

In order to observe the reaction of the rabbit omentum to granular material introduced into the peritoneal cavity, 10 c c. of India ink were injected intraperitoneally. The omentum was removed at the end of 24 hours, fixed and stained with hematoxylin and eosin. It was found that the omentum was jet black, and in some places the ink formed a

thick coating. On microscopic study the ink was found contained chiefly within large phagocytic cells, most numerous in the "taches laiteuses." Similar results were obtained when a 5% suspension of carmine was substituted.

Vital staining of the rabbit omentum was found most satisfactory by the use of 4 subcutaneous injections of 2 c c. of 1% trypan blue at daily intervals. The dye appeared in the general circulation after a period of 15 minutes, stained the serum a deep blue after one hour, and caused a generalized stain at the end of 24 hours. The preparations revealed a characteristic distribution of the dye. The serosal lining cells took on little stain, the fibroblasts more, and the clasmatocytes most, the latter sometimes being loaded with the blue granules. The fibroblasts were usually branched, and the blue granules could be traced into the various prolongations. The clasmatocytes were somewhat elongated or oval in shape and were found chiefly in the "taches laiteuses."

Experiments on the method of phagocytosis by the omentum were made next. One consisted in the introduction of 20 c c. of a mixture, containing 10 c c. of a 5% suspension of carmine in 25% acacia in normal salt solution and 10 c c. of defibrinated chicken blood, into the peritoneal cavity of several rabbits. The omentum was removed at the end of 24 hours, fixed and stained in the usual manner. It was found that the corpuscles and carmine granules were taken up in large quantities by the phagocytic cells found chiefly in the "taches laiteuses." Some of the clasmatocytes contained from 8 to 12 nuclei of the chicken corpuscles. The cell bodies of the erythrocytes became indistinct, although there were many "ghosts" left within the cytoplasm of the phagocytes. The second procedure utilized rabbits which had been vitally stained with trypan blue and then received a suspension of chicken corpuscles in acacia mixture. The omentum removed at the end of 24 hours showed a picture similar to that obtained with the first method, but the clasmatocytes contained both blue granules and numerous chicken nuclei.

Since the rabbit omentum takes up particles from the peritoneal cavity, it was decided to study this process at frequent intervals after the introduction of chicken blood. Several rabbits were vitally stained with trypan blue. Then 10 c c. of a mixture containing 5 c c. of defibrinated chicken blood and 5 c c. of a 25% acacia suspension were injected into each peritoneal cavity. The omentum was removed at the end of 12,

24, 48 and 96 hours, was fixed and stained in the usual manner. This study showed that at the end of 12 hours there was practically no fluid in the peritoneal cavity, but that many smaller and larger clumps were adherent to the omentum. The stained preparations showed many chicken red cells adherent to the external surface of the omentum, and a few intact erythrocytes contained within the vitally stained phagocytic

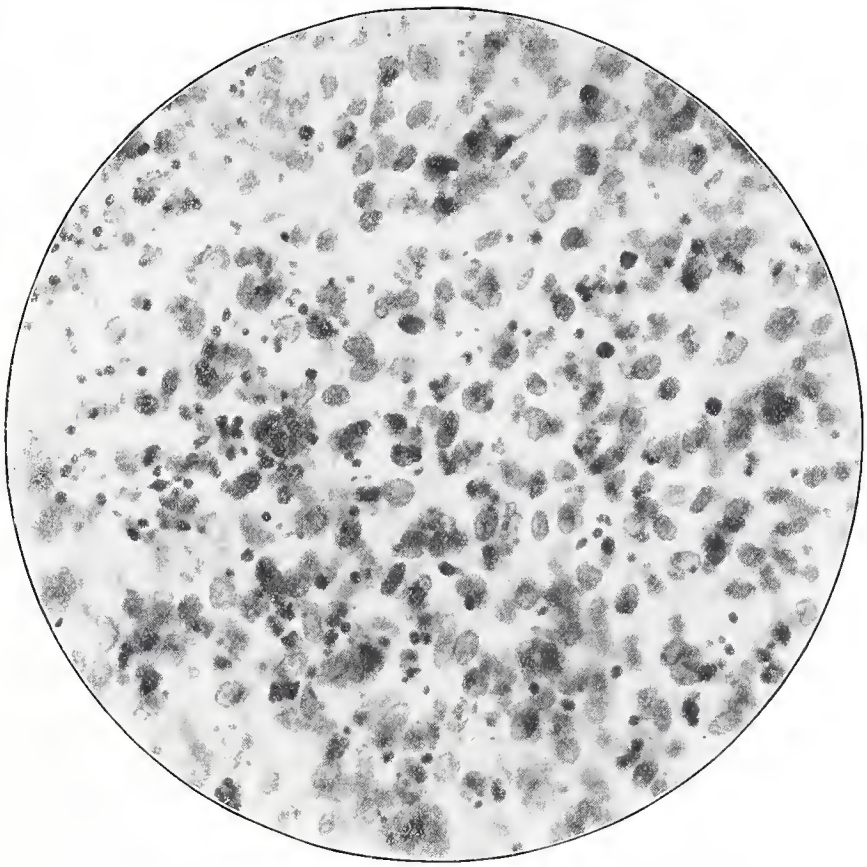

Fig. 5.—Higher magnification of the cells in an omentum removed 24 hours after injection of chicken blood, illustrating the method and degree of phagocytosis by the clasmatocytes. Many of the chicken corpuscles are not as yet completely taken up and are seen as small dark nuclei surrounded by an indistinct rim of cytoplasm. × 350.

cells in the "taches laiteuses." However, at the end of 24 hours these cells were loaded with the nuclei from the chicken corpuscles, while the cell bodies could no longer be recognized (fig. 5). After 48 hours there was considerable fragmentation of these nuclei, so that they were almost unrecognizable. At the last stage, there was considerable granular material contained within the cytoplasm of the cells.

The same experimental method was carried out on another group of vitally stained rabbits, except that in this set the rabbits had received 5 c c. of defibrinated chicken blood 12 days previous to the second injection of 10 c c. of a mixture containing 5 c c. of defibrinated chicken corpuscles and 5 c c. of a 25% acacia suspension. The omentum was removed at the end of 6, 12 and 24 hours. After 6 hours, about 7 c c.

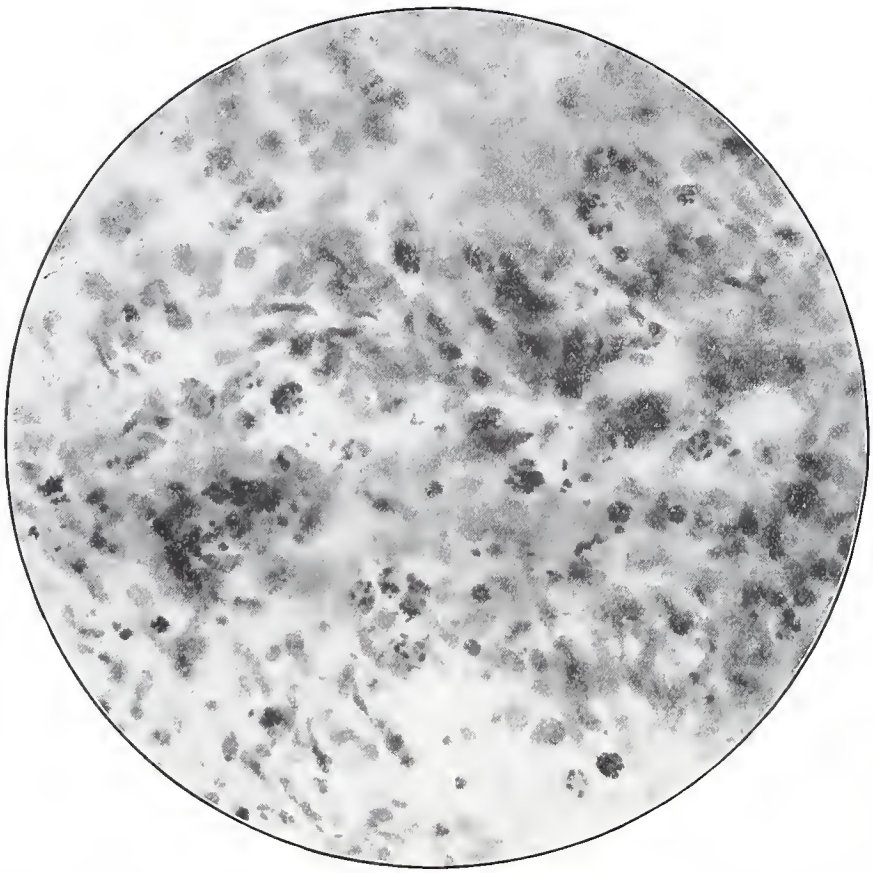

Fig. 6.—Similar to fig. 5, except that the omentum was removed at the end of 6 hours from a rabbit which was at the height of immunity. This structure exhibits a more advanced and complete degree of phagocytosis although observed 18 hours earlier than the nonimmunized rabbit. × 350.

of fluid deeply stained with laked blood were found free in the peritoneal cavity. This exudate contained "ghosts" of corpuscles, swollen and edematous nuclei, and an occasional intact red cell. The preparations of the omentum showed the clasmatocytes loaded with the chicken corpuscle nuclei and containing numerous blue granules (fig. 6). The animals studied after 12 and 24 hours contained a similar amount of

blood tinged fluid, only there were no red cells and few intact nuclei in the smears of the exudate. The omentum showed a more advanced degree of phagocytosis and earlier fragmentation of the nuclei than that seen in the nonimmunized animals. Intraperitoneal hemolysis was present. It was also noted that the clasmatocytes in these preparations contained more trypan blue and seemed more numerous than in the rabbits which had received only one injection of the blood.

In order to determine the relation of this increased vital staining to functional activity, 10 c c. of defibrinated sheep blood were injected into the peritoneal cavity of several rabbits, and omentum removed on the 4th, 8th, and 12 days. Each animal received 4 daily injections of trypan blue prior to removing the omentum. This structure was fixed and stained in the usual manner. This study showed that the omentum, at the end of 4 days, took on more stain than the controls, and the "taches laiteuses" were more prominent. At the end of 8 and 12 days, there was an increased number of clasmatocytes, increased vascularity of the cellular islands and more intense vital staining of the clasmatocytes. It would appear that the ability of the macrophages to take on trypan blue might be used as an index of functional activity. Similar experiments were carried out with antigen injected intravenously. The omentum removed at varying stages of antibody production showed a slight increase in the number of clasmatocytes in the "taches laiteuses" with some increase in the vital staining. The same type of experiments were made with sheep serum, and similar results were obtained.

Dog Omentum.—The omentum of the dog is an extensive, apron-like membrane (fig. 7). The main structure consists of a dense fibrous network containing a large amount of adipose tissue and large blood vessels. The membranous part between these supporting tissues is semitransparent. The vessels form extensive anastomoses throughout, but there is a conspicuous absence of the cellular islands seen in the rabbits. There are small cellular aggregations of phagocytes along the vessels. The omentum was studied at the height of antibody production, but there were no definite discernible reactions.

Guinea-Pig Omentum.—The omenta from many guinea-pigs revealed a rather uniform picture (fig. 8). This structure measured about 8 by 3.5 cm., was a thin membrane, except along the insertion to the greater curvature of the stomach. The groundwork consisted of interlacing intercellular processes surrounding vacuoles of varying size. The nuclei of the cells were oval or round, although some were

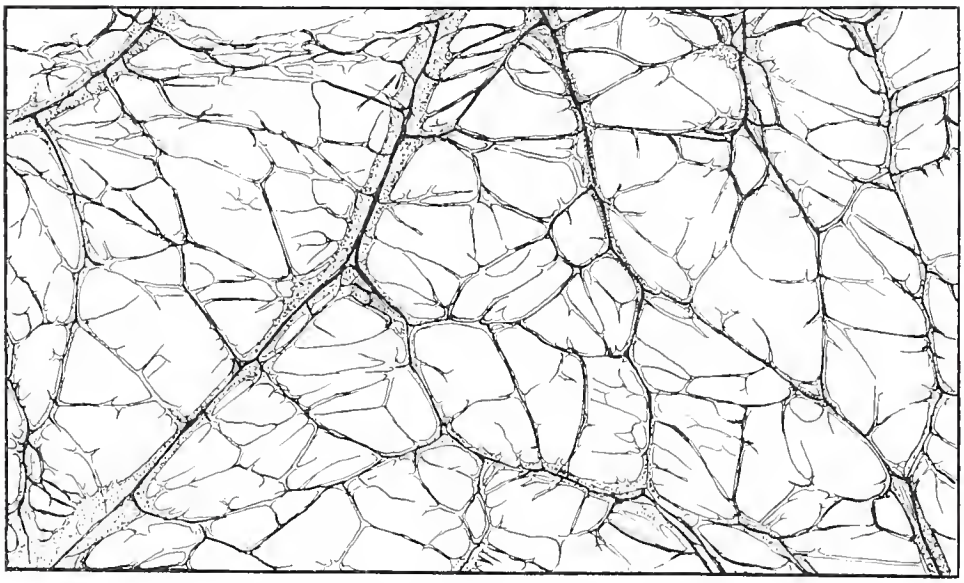

Fig. 7.—Sketch of the dog omentum, showing the characteristic gross appearance of this membrane with its vascular framework and absence of the phagocytic cells.

Fig. 8.—Sketch of the guinea-pig omentum, demonstrating its general structure with the numerous glomerulus-like bodies scattered along the blood vessels.

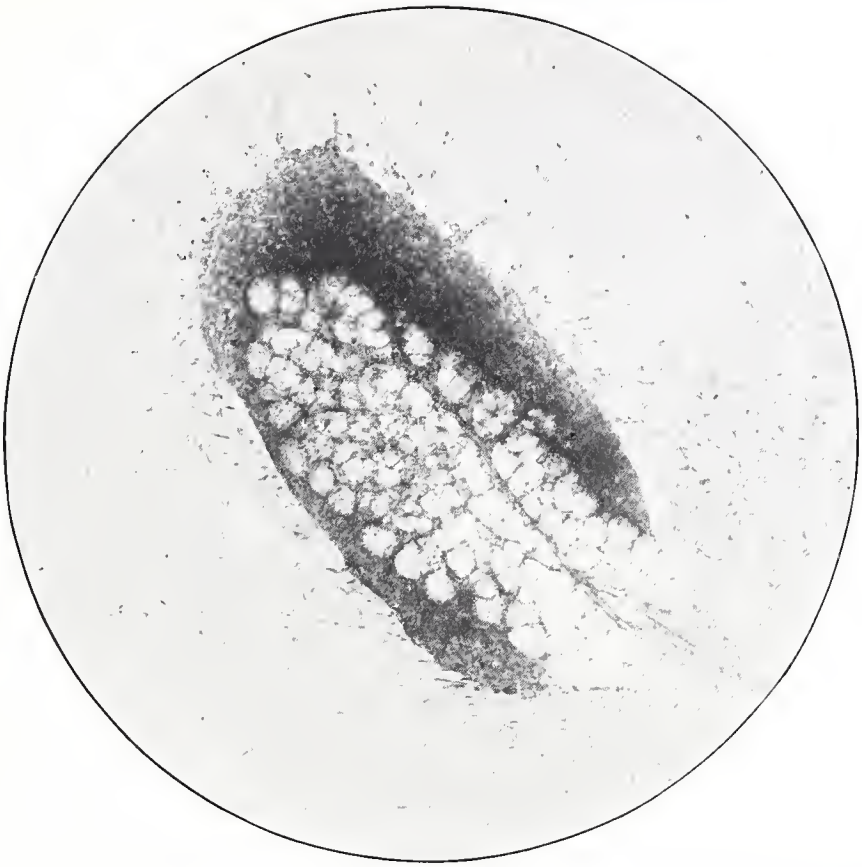

Fig. 9.—Low power representation of a glomerulus-like body in guinea-pig omentum, showing the characteristic central capillary network and the peripheral cellular aggregations. × 60.

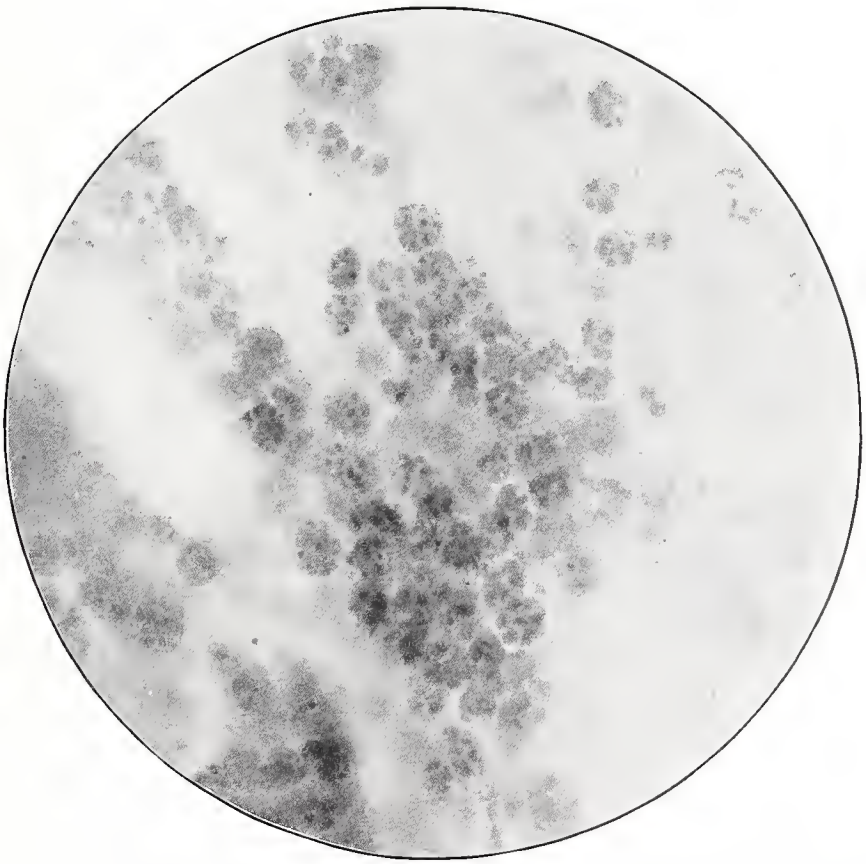

Fig. 10.—Higher power representation of the marked phagocytosis of chicken corpuscles by the cellular constituents of the glomerulus-like bodies of the guinea-pig omentum removed after 24 hours. × 350.

elongated and spindle shaped. The larger vessels had a fibro-fatty supporting tissue, in which were embedded many small accumulations of phagocytic cells. Many of the terminal vessels ended in a glomerulus-like structure supplied by one or two small arterioles and drained by one vein. The peripheral part of these bodies was usually made up of cellular aggregations (fig. 9).

Trypan blue was injected into several guinea-pigs. The omentum was removed, fixed and stained. It was found that the cells took out only a small amount of stain from the general circulation, and this was well confined to the cells composing the glomerulus-like bodies. Chicken corpuscles were injected into the peritoneal cavities of several vitally stained guinea-pigs. The omentum was removed at the end of 24 hours. There was only a moderate degree of phagocytosis and this chiefly by the phagocytic cells described (fig. 10).

A group of experiments was made similar to those performed in the rabbit. The results obtained corresponded with the lessened ability of the guinea-pig to produce antibodies. The degree of phagocytosis of the immunized animals was somewhat increased over that of the normal, but there was an entire absence of the intraperitoneal hemolysis which occurred in the immunized rabbits. The phagocytic cells seemed to take on only a slightly increased amount of trypan blue at the height of antibody production.

DISCUSSION

The early experiments demonstrated that rabbits deprived of their omentum produced, at the height of the antibody curve, about one-fourth the concentration of antibodies in their serum, in response to a single intraperitoneal injection of antigens, of that produced by normal control rabbits. Removal of the omentum soon after the introduction of the antigen also caused a similar decline, but this had little effect when practiced later than the eighth day after the onset of immunization. Rabbits without the omentum did not show any decrease in antibody production after intravenous injection of the antigens. Sheep blood and serum, and chicken blood were used as the antigens. The results were similar with both the cellular and soluble antigens but not as marked with the latter. The antibody production in dogs and guinea-pigs was independent of the presence of the omentum following both intraperitoneal and intravenous injection of the antigens. In rabbits, splenectomy when combined with the removal of the omentum produced a more decided decrease in the subsequent concentration than

simple removal of either organ. Omental transplants did not remain viable and had no influence on the antibody production in animals which received this tissue at the time of injection of the antigen.

It was found advisable, because of this discrepancy resulting from intraperitoneal and intravenous injections of the antigens in rabbits and of the species differences, to make a careful morphologic study of the various omenta, and as stated it was found that the omentum in the three groups has an entirely different structure. The rabbit has a well developed organ with numerous "taches laiteuses," while the omentum of the guinea-pig with its glomerulus-like bodies stands next, and finally comes that of the dog, which is nearly devoid of well defined phagocytic cellular islands. The vascularity of the three also shows various gradations; the dog being most vascular, the rabbit next, and the guinea-pig least. These gross differences were further emphasized by detailed study of the individual cell types in their reactions to foreign material and blood introduced into the peritoneal cavity, and to vital stains.

The rabbit omentum readily takes up granular and cellular materials from the peritoneal cavity. The latter reaction is shown clearly by the use of chicken corpuscles, which because of their nuclei can be distinguished with ease. The most characteristic feature, however, is that the most marked phagocytosis occurs in the cells comprising the "taches laiteuses." It was noted further that this process is greatly accelerated by previous immunization. The injected foreign blood also becomes laked in the peritoneal cavity of the immunized rabbit, a phenomenon not observed in the control rabbits. The guinea-pig omentum, likewise, takes up the chicken corpuscles chiefly in the cells which are situated about the periphy of the glomerulus-like bodies. This phagocytic activity is not much increased by previous immunization, and no intraperitoneal hemolysis of the foreign corpuscles ensues. The omentum of the dog exhibits little phagocytosis, and this property is not augmented by previous immunization.

Cunningham has demonstrated that the three cell types of the rabbit omentum can be differentiated by injections of trypan blue as a vital stain. My omental preparations confirm these results, as the cells of the serosal lining take on little dye and have a pavement-like arrangement forming two distinct layers. The fibroblasts are elongated branched cells, scattered throughout the membrane but most numerous along the blood vessels and in the region of "taches laiteuses." These

cells contain much more trypan blue than the serosal lining cells, and frequently contain granules extending out into intercellular processes. The clasmatocytes react in a characteristic manner. They are found chiefly in the cellular islands, frequently filled with blue dye and either oval or round, with a somewhat vesiculated nucleus. These cells are the chief phagocytic elements of the omentum and often have from 8 to 10 nuclei of chicken erythrocytes 48 hours after injecting the blood. The clasmatocytes at the height of immunity show a more intense degree of vital staining and phagocytize the corpuscles more readily and completely. Similar cells are also found scattered throughout the membrane. This increase in vital staining of the clasmatocytes becomes progressively more intense between the initial injection of the antigen and the height of the antibody curve, which occurs about the 12th to 14th day. The guinea-pig omentum contains similar phagocytic cells about the periphery of the glomerulus-like bodies. This cell type also takes on vital stain the most intensively of the cellular constituents of the structure, but does not demonstrate a definitely increased staining capacity during the course of antibody production.

The relationship between phagocytosis and antibody production could not be definitely established. The rabbit with its highly developed phagocytic activity produced a high concentration of antibodies, and when deprived of the omentum with its numerous clasmatocytes, had its antibody function restricted. The dog with little similar phagocytic reaction produced antibodies comparable to those of the rabbit, but did not produce the same variety of antibodies in response to the same antigen. The results also showed that removal of the omentum from the dog with its few phagocytic cells has no lowering effect on the subsequent course of antibody production. The differences in the responses in the species may be a quantitative variation in the number of local clasmatocytes stimulated at the site of injection of the antigen and possibly a qualitative difference, as precipitins are not formed in the dog in response to injections of sheep blood. The early morphologic study of the guinea-pig omentum seemed to indicate that this structure should be between that of the rabbit and the dog, as there is a well defined phagocytic activity in the structures similar to the "taches laiteuses" of the rabbit omentum. However, the guinea-pig produces little soluble antibodies, and these in a low concentration. The difference between the guinea-pig and the rabbit is evidently a qualitative one. The differences between the guinea-pig and the dog are quantitative

as to the number of the phagocytic cells and qualitative as to the variation of antibody reaction to the same antigen. The clasmatocytes of the rabbit omentum seem to be a definite factor in antibody production in this species, because the removal of the omentum reduces the antibody-producing ability of the rabbits after intraperitoneal injection of the antigen; because of the increased phagocytosis by these cells at the height of immunity; and because of the occurrence of intraperitoneal hemolysis of foreign corpuscles in immunized animals.

Cunningham states that the variation in the storage of vital dyes in the cells represents corresponding differences in physiologic adaptation. The increased vital staining of the clasmatocytes at the height of antibody production probably indicates, then, a greater functional activity of these cells, possibly in producing antibodies.

SUMMARY

The omentum of the rabbit takes part in the development of antibodies in response to intraperitoneal injection of antigen, but in the dog and guinea-pig the omentum seems far less active in this respect.

The differences are probably due to variations in the structure of the omentum of the three species. The rabbit omentum has well defined "taches laiteuses" consisting chiefly of clasmatocytes, that of the dog has only few cellular islands, while the guinea-pig omentum contains glomerulus-like bodies, but this animal has little power to produce free antibodies.

The omentum of the rabbit shows greater activity at the height of antibody production, as evidenced by increased phagocytic ability and more intense degree of vital staining of the clasmatocytes, and intraperitoneal lysis of foreign corpuscles.

Transplanted rabbit omentum soon is converted into a fibrous tissue devoid of endothelial cells.

The clasmatocytes of the rabbit omentum appear to be a definite factor in antibody production following intraperitoneal injection of antigen.

CONDUCTIVITY OF YEAST CELLS

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In previous papers [1] from these laboratories there has been reported findings on the conductivity of bacterial cells. New methods were introduced in that the bacterial cells were suspended in salt solutions of various strengths and resistance measurements made of the suspending menstruums as well as of the suspensions. This led to the findings that dead bacterial cells offer considerable electrical resistance, which was contrary to the previous observation of Shearer.[2] It was demonstrated that bacterial cells store up within their bodies diffusible salts in a concentration greater than is found in the nutrient medium, and that on death these salts pass out into the menstruum if such is of a lesser salt concentration.

These diffusible salts were termed the physiologic salts in contradistinction to the nondiffusible, which were termed the structural salts. The results were substantiated by a subsequent quantitative analysis of the diffusible and nondiffusible salts reported by Guillemin and Larson.[3]

In view of the great variation in the results and conclusions of investigators making conductivity measurements on various tissues and cells, it has appeared of importance to apply the experimental methods previously used on bacterial cells to yeast cells. The following report deals with experiments on the conductance of suspensions of Saccharomyces cerevisiae.

The yeast cells were seeded on Kolle flasks containing a modified Sabouraud agar made up with 1% dextrose and 1% sodium chloride. After 20 hours the growth was washed off with distilled water, and the suspension centrifuged. The supernatant liquid was drawn off and discarded. The packed cells were then divided into two or more equal portions by weight, and each portion was washed twice in the strength of salt solution which was to be used in the final experimental procedures.

The final suspensions were made by adding salt solutions by volume to give two or more suspensions containing the same amount of yeast

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[1] Papers from Mayo Foundation for Medical Education and Medical School, 1915-1920, p. 577; Jour. Infect. Dis., 1922, 30, p. 550.

[2] Jour. Hygiene, 1919, 18, p. 360.

[3] Jour. Infect. Dis., 1922, 31, p. 349.

cells and differing only in the electrolyte content of the suspending liquid. The total resistances of the suspensions were then measured. By centrifuging, the menstruums were separated and their resistance values determined. The suspensions were again formed by a thorough mixing, and the suspension subjected to lethal heat.

The resistances were then determined for the heated suspensions and menstruums. The measurements were made in an electrolytic cell having a volume of about one and a half mils. An alternating current of 4,000 cycles was used, which was obtained from a Vreeland oscillator. The data in the following experiments are given as measured resistances

TABLE 1

DEATH OF YEAST CELLS IN NACL SOLUTIONS

Yeast Cells.	Resistance per cm³ in Ohms		
0.25% NaCl			
Suspension alive	241.5	Suspension dead	165.2
Menstruum alive	197.6	Menstruum dead	151.2
0.50% NaCl			
Suspension alive	137.0	Suspension dead	109.3
Menstruum alive	110.0	Menstruum dead	96.6

TABLE 2

DEATH OF YEAST CELLS IN NACL SOLUTIONS

Yeast Cells.	Resistance per cm³ in Ohms		
0.25% NaCl			
Suspension alive	224.7	Suspension dead	173.4
Menstruum alive	198.4	Menstruum dead	161.0
0.50% NaCl			
Suspension alive	128.5	Suspension dead	108.4
Menstruum alive	109.3	Menstruum dead	98.6

divided by the cell constant, or resistances of centimeter cubes of menstruums and suspensions. Reference to cells and menstruums before heating is indicated by the term "live" and after heating by "dead." "Menstruum dead" indicates the liquid suspending the cells during and after subjection to heat.

In tables 1 and 2 are given data on the death of yeast cells in NaCl solutions. In each case the yeast cells were divided into two equal parts, and each part washed and suspended in 0.25% and 0.50% NaCl solution, respectively. Measurements of the menstruums and suspension resistances were made, and the suspensions were heated at 80 C. for 15 minutes. After centrifuging, the resistances of the menstruums dead were determined, and finally the resistances of the suspensions dead.

The suspensions used for data in table 2 were not as heavy suspensions as were used for table 1. Graphic representations of these data are given in fig. 1 and fig. 2.

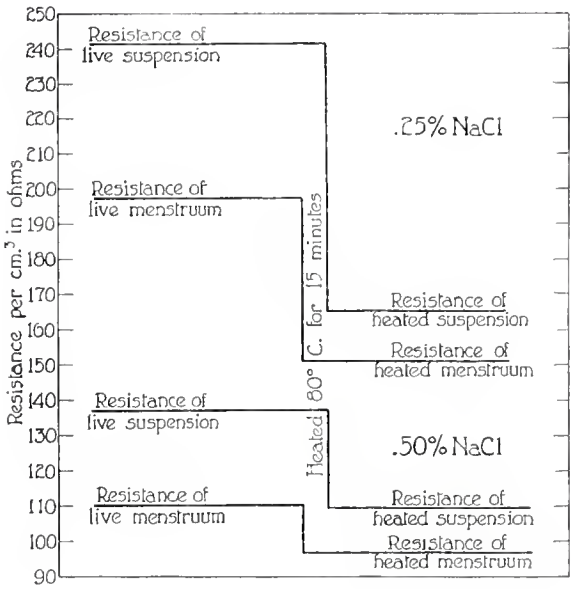

Figure 1.

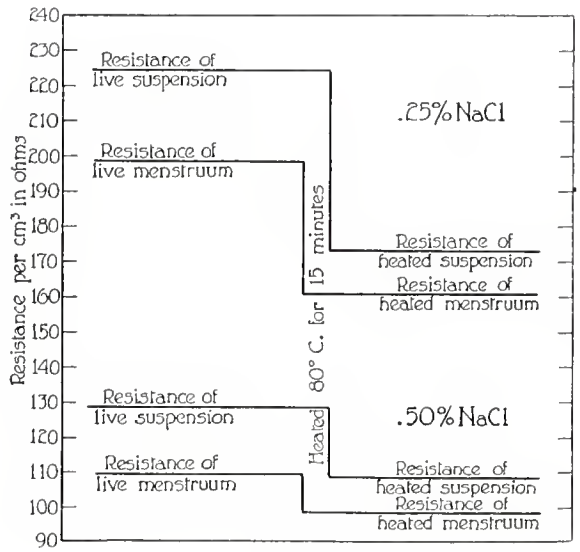

Figure 2.

It is to be noted that the heavier suspensions of table 1 raised the resistance of the menstruums the greater amount. On heating, the resistances of the suspensions decreased and also the resistances of the menstruums decreased.

The dead yeast cells raised the resistances of the menstruums appreciably when suspended in them but apparently not to as great an extent as did the live cells. The decrease in the resistances of the menstruums can be definitely referred to an exosmosis of electrolytes from the cells as reported by Green,[1] and more recently by Brooks.[4]

As the magnitude of the foregoing changes in resistance is apparently related to the electrolyte concentration of the menstruum, similar procedures were carried out utilizing a large range of electrolyte concentration, and these data are given in table 3.

It is evident from the foregoing that the magnitude of the resistance offered by the yeast cells is dependent upon the strength of the electrolyte suspending them. Likewise, the magnitude of the drop in the

TABLE 3

DEATH OF YEAST CELLS IN NaCl SOLUTIONS

Conc. NaCl Percentage	Yeast Cells.	Resistance per cm³ in Ohms. Living		Dead
0.1	Suspension	570.4	Suspension	187.8
	Menstruum	355.0	Menstruum	157.1
0.2	Suspension	368.2	Suspension	159.1
	Menstruum	231.8	Menstruum	130.5
0.5	Suspension	162.5	Suspension	104.6
	Menstruum	101.9	Menstruum	84.0
0.6	Suspension	143.5	Suspension	94.7
	Menstruum	87.7	Menstruum	77.3
0.9	Suspension	95.4	Suspension	73.3
	Menstruum	61.2	Menstruum	59.2
1.0	Suspension	84.9	Suspension	66.7
	Menstruum	55.3	Menstruum	54.8

resistance of the menstruum is dependent on this factor. The greater the concentration of the surrounding electrolyte, the less is the exosmosis of salts from the cells and likewise the less is the measurable effect of those salts which do diffuse. If the surrounding electrolyte is in greater concentration than is found inside the cell, salts will rapidly diffuse into the cells on death, giving a rise in resistance of the menstruum as others of our experiments have demonstrated. When no change takes place in the resistance of the menstruum on death of the cells, the menstruum concentration probably represents the concentration of the physiologic salts inside the cell. As is seen in table 3, there was diffusion of electrolytes into the 1% NaCl menstruum, which indicates that yeast cells store up in their bodies diffusible salts to a greater concentration than is found in the medium on which they grow.

[4] Jour. Gen. Physiol., 1923, 5, p. 365.

As pointed out by Green and Larson,[1] there are 3 factors concerned with the drop in resistance of cellular suspension on death: exosmosis of salts, decrease in size of cells with a resultant decrease in ability to displace a better conducting medium, and increased conductance of the cells. The foregoing data demonstrate that the exosmosis of salts is an important factor in conductivity measurements of yeast cells.

Direct measurements which we have made on yeast cells, with the use of positive and negative stains, indicate that there is a marked decrease on death in the size of yeast cells, which is greater in proportion than that observed with bacterial cells. Decrease in size of cells is then also a factor in the large decrease in resistance observed on heating a suspension of yeast cells. If we could evaluate these factors, some idea could be obtained as to the actual increase in permeability of the cells—the real question in point.

In attempting any such evaluation from data similar to that presented here it is necessary that certain simplifying misconceptions be avoided. It is to be pointed out that a subtraction of the resistances of the menstruums from that of the suspensions does not give a value for the resistance of the cells but simply the amount which the resistance was increased by displacing part of the menstruum by a less conducting medium—the cells. A direct subtraction indicates resistances in series which is not the case in such a system, as the current has a choice of paths in passing through the suspension. This was recognized and indicated by Green and Larson.[2] Also in comparing the increase in resistance brought about by adding live and dead cells to a menstruum, it is necessary to take into account the conductivity of the menstruum. In the 0.25% NaCl concentration of table 1, the data gives by subtraction 43.9 ohms for the increase in resistance due to live yeast cells and 14 ohms for the increase in resistance due to same cells after heating. These values are not comparable directly as they are based on different electrolyte concentrations of the menstruums. By reference to table 3, it is seen that the same bacteria alive raise the resistance of different strength salt solutions different amounts. It is therefore necessary to base values for the live and dead cells on a common strength salt solution before any comparable evaluation may be attempted. Approximating this by simple proportion for the example referred to above we get a value of 21.6 ohms for the increase in resistance due to dead yeast cells, which is quite different from the value 14 ohms obtained by direct subtraction. If such a calculation is carried out on the data of Green and Larson[1] for B. coli, there is practically no difference between the

effect of dead and live bacterial cells. In the case of yeast cells there is even with this correction a large difference between the ability of live and dead cells to raise the resistance when displacing menstruum. This difference still contains, however, the decrease in resistance dependent on a decrease in the size of the cells on death, a factor which we have found in the case of yeast cells to be large. While accurate computation of this factor is necessary before a definite value can be ascribed to a change in the conductivity of cells from this type of data, the existence of such a change may be inferred. Such consideration tends to show that unlike bacterial cells, yeast cells do assume an increased conductance on death. The difficulties attending even an approximate evaluation of this increase in conductance and the concomitant occurrence of other phenomena make it appear that measurements of total resistances of similar systems may indicate but do not measure cell permeability.

THEORY OF ELECTRICAL CONDUCTANCE OF SUSPENSIONS

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Electrical conductivity measurements have become widely used as a means of studying properties and reactions of living cells. Many far reaching conclusions have been drawn from the results of this type of investigation. Up to the present time little consideration seems to have been given to the fundamental aspects of the problem. All electrical conductivity measurements of living cells represent in final analysis the measurements of the resistance of a system consisting of cellular elements bathed in an electrolyte solution. Such a system when considered even in its simplest form represents a complex combination of resistances. In a previous paper by Green,[1] the importance of considering separately the individual resistances concerned in such a system was pointed out, and such consideration established findings quite at variance with the interpretations of those investigators who have confirmed their data to the measurements of the total resistance of such a system. It has seemed worth while to examine this problem carefully and to determine, if possible, the significance of results which have been obtained by measuring the electrical conductance of inhomogeneous systems.

The first attempts to analyze the resistance of a suspension of cells in an electrolyte solution into its component parts seem to have been made by Stewart,[2] Roth,[3] and Burgarsky and Tang [4] in determining the relative volume of blood corpuscles and serum in defibrinated blood by conductivity measurements. The simple proportionality which was deduced by these investigators was based on a consideration that the cells were nonconductors in comparison with the good conducting serum. The problem was later taken up by Oker-Blom [5] who considered the electrical system in some detail. He considered the possible arrange-

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[1] Papers from Mayo Foundation for Medical Education and Research and Medical School, 1915-1920, p. 577.

[2] Jour. Boston Soc. Med. Sc., 1897, 1, p. 18

[3] Elect. Leitfahigkeit thiescher Flussigkeiten. 1897.

[4] Ztschr. f. Physiol., 1897, 2, p. 297.

[5] Pflüger's Arch., 1900, 79, p. 111, 510.

ments of resistances in such a system, depicting the arrangement as resistances in series and also in parallel. He attempted to throw some light on the problem by carrying out experiments on the conductance of suspensions of quartz in salt solutions using 61 parts quartz and 39 parts liquid, and he offered many empirical formulas to express his findings.

The later work of Stewart [6] on hemolysis and that of McClendon [7] on the changes in electrical conductivity of echinoderm eggs on fertilization stimulated the application of conductivity measurements to the study of functional changes in cells. Osterhout [8] has carried out extensive measurements on masses of laminaria tissues wet with sea water, with special reference to injury and salt antagonisms. Gray [9] performed similar experiments with sea urchins' eggs suspended in sea water, and Shearer [10] has used suspensions of bacteria in Ringer's solution. The later work gives little consideration to the fundamental nature of the systems investigated as a combination of resistances, but is concerned mainly with measurements of total resistances. Changes in the total resistance of systems studied have been regarded simply as a measure of a change in that particular member of the system concerning which information was desired.

As cell conductivity measurements are carried out by measuring the resistance of the cells while bathed in an electrolyte solution, the total resistance represents at least two individual resistances, that of the solution and that of the cells. This is the case in measurements of suspensions of free living cells. When mass tissue is used, the system is probably more complex, as in addition there is present an intercellular substance to complicate the picture. In his later work, Osterhout [8] has proposed that in resistance measurements of laminaria disease he was dealing with two resistances, the protoplasm and the cell wall, which were resistances connected in parallel. He assumed a fixed value for one of these resistances and calculated the second on that basis. We fail to see in his reports any evidence to warrant the value assumed or any justification for assuming the value constant, as this simply introduces calculations to give a new series of results of the same order of significance as the originally measured total resistances. More recently,

[6] Jour. Pharm. Exper. Therap., 1910 1, p. 49.
[7] Am. Jour. Physiol., 1910, 27, p. 270.
[8] Jour. Gen. Physiol.. 1921, 4, p. 1.
[9] Proc. Roy. Soc. Med., 1916, 207, p. 481.
[10] Jour. Hygiene, 1919, 18, p. 360.

Green and Larson [11] have reported conductivity measurements on suspensions of bacteria which are representative of a more simple system of resistances, and they point out that the total resistance of such suspensions does not indicate directly the value of the individual resistances concerned. They further assert that changes in the system on death of the cells involve several factors, including exosmosis of salts, decrease in the size of the cells as well as changes in permeability, if such occur. They indicated that the total measured resistances were not a direct measure of an increase in permeability of the cell membranes on death of the bacteria. The exosmosis of salts into the surrounding medium has been verified by the quantitative analysis of these salts reported by Guilleman and Larson [12] and also by the recent measurements of Brooks.[13]

Consideration of conductivity measurements as a system of resistances has recently been reported by Brooks,[14] and this represents the most complete fundamental study up to the present time. Details of his developments have not yet been published, but he takes into account two possible arrangements of the resistances concerned, one arrangement in series and one in parallel. He considers values for the resistance of cells calculated on this basis as limits between which the true value must lie.

To consider the fundamental aspects of electrical conductivity measurements of cells, we have chosen the most simple type of system used in such investigations. This is represented by a suspension of free cells in an electrolyte solution. In such a system there is no intercellular substance except the suspending menstruum, and this can be separated by centrifuging and its value as a component of the system determined. Even for such a relatively simple case as this the problem is one of great difficulty. The fact that an alternating current is generally used in determining the effective resistance adds to the difficulty of an exact interpretation. In this study, however, we assume that the effective resistance as measured by an alternating current is the same as would be obtained using a steady direct current.

In a rather simple manner we can arrive at a solution which we will later show to be an excellent first approximation to an exact solu-

[11] Jour. Infect. Dis., 1922, 30, p. 550.
[12] Ibid., 1922, 31, p. 349.
[13] Jour. Gen. Physiol., 1923, 5, p. 365.
[14] Paper read before Federation of Am Soc. for Exper. Biol. at Toronto, Dec., 1922.

tion. Let us suppose we are dealing with a bacterial suspension or any similar system. We shall use the following symbols:

R = resistance of suspension
M = resistance of menstruum in the electrode cell by itself
C = electrode cell constant
k = specific resistance of menstruum
s = specific resistance of suspended cells
a = fraction of total volume of suspension occupied by suspended cells

The resistance vessel is equivalent then to one which is C cm. long and has a uniform cross section of 1 cm.^{2}

In 1 cm.^{3} of suspension we have (1 — a) c c. of liquid. Hence the cross-section of liquid is (1 — a) cm.,^{2} and the average cross-section of suspended cells is *a* cm.^{2} On this view, 1 cm.^{3} of suspension contains two conductors in parallel, each 1 cm. long, but of different cross-sections, viz., 1 — a and *a* cm.,^{2} respectively; hence, we obtain when the cell is filled with the suspension:

$$\frac{1}{R} = \frac{1-a}{ck} + \frac{a}{cs} = \frac{1-a}{M} + \frac{a}{cs}$$

$$a = \frac{1-\frac{M}{R}}{1-\frac{M}{cs}} = \frac{1-\frac{M}{R}}{1-\frac{k}{s}} \qquad \text{Equation A.}$$

If the resistivity of the suspended cells is infinitely great, we obtain the approximate equation: $a = 1 - \frac{M}{R}$ — Equation B.

If we make measurements with different concentrations of the salt solution in which the cells are suspended, we obtain corresponding values of R and M, which we represent by R_1, M_1, R_2, M_2, etc. If we assume that in solutions which differ in concentration both *a* and *s* remain unchanged, we arrive at the following:

$$\frac{a}{cs} = \frac{1}{R_1} - \frac{1-a}{M_1} = \frac{1}{R_2} - \frac{1-a}{M_2} \text{ and } 1-a = \frac{\frac{1}{R_1}-\frac{1}{R_2}}{\frac{1}{M_1}-\frac{1}{M_2}} = \frac{M_1 M_2 (R_2 - R_1)}{R_1 R_2 (M_2 - M_1)}$$

$$a = 1 - \frac{M_1 M_2 (R_2 - R_1)}{R_1 R_2 (M_2 - M_1)} \qquad \text{Equation C.}$$

In applying equation C, only experimental data should be used in which the values of $(R_2 - R_1)$ and of $(M_2 - M_1)$ are relatively large; otherwise the relative errors in these terms will render the expression useless.

A somewhat more rigorous treatment of the subject may now be given. Assuming that the suspended cells are all spheres of equal radii; imagine the volume of liquid divided into as many equal cubes as there are spherical cells. Let l = length of an edge of the cube and let r = radius of each sphere. We can readily calculate a lower limit [15] to the resistance of a cube of material whose resistivity is k enclosing a sphere whose resistivity is s by assuming that the lines of flow of current are all parallel and in our case normal to two opposite faces of the cube. With this assumption, the sphere may occupy any position inside the cube. As a matter of fact, the lines of flow of current will not be parallel but will tend to avoid the sphere if the latter has a greater resistivity than the surrounding medium. Nevertheless, it may be of interest to calculate the resistance on the basis of this simplifying assumption. For a distance $(l - 2r)$ we have to calculate only the resistance of the liquid, while through a distance $2r$ we have a cross-section which is partly surrounding liquid and partly material of the sphere. It might be added at this point that we are assuming that the sphere is uniform and homogeneous. The resistance of the menstruum then for a distance $(l - 2r)$ is $\frac{k(l-2r)}{l^2}$. As regards the remaining portion, let us take our origin at one end of a diameter of the sphere which is parallel to the lines of flow. Let us take a lamina of thickness dh normal to the lines of flow and at a distance h from the origin, the cross-section which lies in the sphere will have an area equal to $2\pi rh - \pi h^2$, the cross-section outside the sphere will have an area equal to $l^2 - 2\pi rh + \pi h^2$. The resistance of each part of the lamina will be $\frac{sdh}{2\pi rh - \pi h^2}$ and $\frac{kdh}{l^2 - 8\pi rh - \pi h^2}$. Since these are in parallel, the resistance of the lamina will be

$$\frac{k \quad s \quad dh}{sl^2 - 2\pi rh\,(s-k) + \pi h^2\,(s-k)}$$

By integrating from $h = o$ to $h = 2r$ we obtain

$$\frac{2ks}{\sqrt{\pi l^2 s\,(s-k) - \pi^2 r^2\,(s-k)^2}} \tan^{-1} \frac{\pi r\,(s-k)}{\sqrt{l^2 s\,(s-k) - \pi^2 r^2\,(s-k)^2}}$$

and therefore for the total resistance of the cube:

$$\frac{k(l^2 - 2r)}{l^2} + \frac{2k}{\sqrt{\pi l^2\left(1 - \frac{k}{s}\right) - \pi^2 r^2\left(1 - \frac{k}{s}\right)^2}} \tan^{-1} \frac{\pi r\left(1 - \frac{k}{s}\right)}{\sqrt{\pi l^2\left(1 - \frac{k}{s}\right) - \pi^2 r^2\left(1 - \frac{k}{s}\right)^2}}$$

[15] Jeans: Electricity and Magnetism, p. 355.

On expanding the inverse tangent function and making the substitution $a = \frac{4/3 s \pi r^2}{l^3}$ we obtain for the total resistance of the cube

$$\frac{k}{l}\left[1 + a\left(1 - \frac{k}{s}\right)^2 + 0.967a^{5/3}\left(1 - \frac{k}{s}\right) + 1.003a^{7/3}\left(1 - \frac{k}{s}\right)^2 +)\right]$$

Accordingly there results

$$R = M\left[1 + a\left(1 - \frac{k}{s}\right) + 0967a^{5/3}\left(1 - \frac{k}{s}\right)^2 + 1.003a^{7/3}\left(1 - \frac{k}{s}\right)^3 + \right]$$

and this is very close to $R = \frac{M}{1 - a\left(1 - \frac{k}{s}\right)}$ or $a = \frac{1 - \frac{M}{R}}{1 - \frac{k}{s}}$ which is our equation A.

If we assume that the suspended cells are cubes instead of spheres, we arrive readily at the relation

$$R = M\left[1 + a\left(1 - \frac{k}{s}\right) + a^{5/3}\left(1 - \frac{k}{s}\right)^2 . .\right] = M\left[1 + \frac{a\left(1 - \frac{k}{s}\right)}{1 - a^{2/3}\left(1 - \frac{k}{s}\right)}\right]$$

This is identical with the formula for the lower limit of resistance contained in a recent letter from Dr. S. C. Brooks, if we substitute in our formula $n = 1000a$ and $k = \frac{(s)}{(k)}$.

In obtaining equation C we assumed two sets of measurements, M_1, R_1 and M_2, R_2, in which *a* and *s* have constant values. We have attempted to fulfil these conditions experimentally and to apply experimental data to the expression obtained. To this end we have made one set of measurements on a bacterial suspension, then changed the salt concentration of the menstruum and carried out a second set of measurements. This procedure has given a constant percentage of cellular substance present and therefore kept *a* constant except in so far as the volume of a cell many change. The same cells were used in both measurements, and this, we assume, gives us a constant specific resistance of the cells in both cases. Brooks [13] believes the resistance of cells changes with a change in the resistance of the menstruum. Our data do not indicate this, but if such is the case our results represent a value for the resistance of bacterial cells dependent on the strength of electrolyte used in the menstruum.

The preliminary experiments reported here were made with B. coli and yeast cells. The measurements of resistance were made with a

modified wheatstone bridge, using an oscillating current of 4,000 cycles obtained from a Vreeland oscillator. The measurements were made in a water bath with temperature constant to 1/25 degree C. The organisms were grown in kolle flasks on plain agar. After 24 hours' growth the cells were washed off with distilled water, centrifuged, rewashed with distilled water by centrifuging and concentrated in a small amount of distilled water to make a heavy suspension. Weighed amounts of this heavy suspension were then added to equal amounts of various strengths of sodium chloride solutions to make several suspensions differing only in the sodium chloride concentration of the suspending menstruum. The total resistance of these suspensions was measured in an electrolytic cell having a volume of one and a half mils, giving the various values of R. The suspensions were then centrifuged and the resistances of the cell filled with the menstruum were then obtained, giving the corresponding values of M. The suspensions were then remade and heated for a time above the lethal temperature and values of M and R obtained for the same suspensions when the organisms had been killed. Data obtained are given in the following tables:

TABLE 1

Live Suspensions of B. Coli, March, 1921

Conc. of NaCl	M	R	$1 - \frac{M}{R}$
0.1%	1463	1625	0.100
0.3%	560	645	0.132
0.5%	350	399	0.123
0.7%	263	303	0.132
0.9%	263	314	0.163

The last column of table 1 gives approximate values of *a* calculated by means of equation B. Employing equation C we obtain from pairs of solutions the results given in table 2.

TABLE 2

Pair of Solutions	$a = 1 - \frac{M_1 M_2 (R_2 - R_1)}{R_1 R_2 (M_2 - M_1)}$
0.1% and 0.3%	0.152
0.1% and 0.5%	0.130
0.1% and 0.7%	0.139
0.1% and 0.9%	0.176
0.3% and 0.5%	0.108
0.3% and 0.7%	0.132
0.3% and 0.9%	0.190
0.5% and 0.7%	0.160
Average	0.148

Equation A may be written in the form $a - \frac{Ma}{cs} = a - My = 1 - \frac{M}{R}$ where $y = \frac{a}{cs}$ Considering a and y as two unknowns to be determined from the five values of $1 - \frac{M}{R}$ given in table 1 we arrive at the following system of equations:

$$\begin{array}{l} a - 1463y = 0.100 \\ a - 560y = 0.132 \\ a - 350y = 0.123 \\ a - 263y = 0.132 \\ a - 263y = 0.163 \\ \hline a - 579.8y = 0.130 \end{array} \qquad \begin{array}{l} 1463a - 2140 \times 10^3 y = 146.3 \\ 560a - 313.6 \times 10^3 y = 73.93 \\ 350a - 122.5 \times 10^3 y = 43.05 \\ 263a - 69.2 \times 10^3 y = 34.72 \\ 263a - 69.2 \times 10^2 y = 42.87 \\ \hline a - 901.9y = 0.1179 \end{array}$$

From these, $a = 0.151$, $y = 3.643 \times 10^{-5}$, $cs = 4145$ ohms. Since the cell constant was $c = 3.95$, we have for the resistivity of the live bcateria, $s = 1050$ ohms. The excellent agremeent between the value of a obtained here and that given in table 2 may be noted.

Similar experiments were made with dead bacteria, the results of which are given in the following tables:

TABLE 3

DEAD B. COLI, MARCH, 1921

Conc. of NaCl	M	R	$1 - \frac{M}{R}$
0.1%	1083	1203	0.100
0.3%	495	555	0.108
0.5%	325	362	0.102
0.7%	250	283	0.117
0.9%	253	295	0.142

Calculating as before the value of a by means of equation C, we obtain an average value of 0.125. Proceeding as before, with the equation $a - My = 1 - \frac{M}{R}$, we obtain the following:

$a = 0.127$
$cs = 4570$
$s = 1160$

It should be emphasized that the value of cs or s is subject to considerable error, which may amount to 20%. Nevertheless, the results of our calculations lead us to infer that the dead bacteria are somewhat smaller than the live ones and that the resistivity of the dead bacteria is possibly greater than that of the live bacteria.

It is interesting to compare with these results, some obtained this year with B. coli:

TABLE 4

Suspensions of B. Coli, February, 1923

NaCl	Live M	Live R	Live $1-\frac{M}{R}$	Dead M	Dead R	Dead $1-\frac{M}{R}$
0.1	374.1	404.1	0.074	337.5	366.5	0.079
0.2	284.9	313.0	0.090	265.6	291.1	0.087
0.5	171.7	188.3	0.088	164.9	180.9	0.088
0.6	150.2	166.3	0.097	144.8	159.7	0.093

Equation C gives for the average value of a, $a = 0.108$ (live) and $a = 0.102$ (dead). From the system of equations of the type $a - My = 1 - \frac{M}{R}$, we obtain the following:

a (live) $= 0.106$
cs $= 1361$ and as $c = 2.20$
s $= 619$
a (dead) $= 0.100$
cs $= 1693$
s $= 770$

Once more the dead bacteria are smaller and apparently have a greater resistivity than live bacteria.

Similar experiments were carried out with yeast cells (Saccharomyces cerevisiae). These organisms were grown on agar containing 1% dextrose, and suspensions were made in the same manner as was described above for B. coli. These cells are much more easily centrifuged than B. coli, and the experiments therefore were carried out in a much shorter length of time. Similar data were obtained by several experiments. Data from a representative experiment are given in table 5.

TABLE 5

Suspension of Yeast Cells, January, 1923

% NaCl	Live M	Live R	Live $1-\frac{M}{R}$	Dead M	Dead R	Dead $1-\frac{M}{R}$
0.1	820.8	1078.0	0.239	359.1	412.4	0.129
0.2	462.6	610.0	0.242	292.3	331.9	0.119
0.5	209.7	283.7	0.261	177.3	205.6	0.138
0.6	179.7	240.0	0.251	157.1	183.8	0.145
0.9	123.6	166.3	0.257	117.4	137.8	0.148
1.0	113.9	153.3	0.257	109.4	128.4	0.148

Application of equation C to the data of table 5 gives the following average result; a (live) $= 0.261$; a (dead) $= 0.162$. Forming sets of equations of the type $(a - My) = 1 - \frac{M}{R}$, we obtain a (live) $= 0.260$; a (dead) $= 0.168$.

$$\text{Resistivity of live cells} = \frac{9300}{c} = 4222.$$

$$\text{Resistivity of dead cells} = \frac{1200}{c} = 545.$$

These calculations from the above data on yeast cells lead us to infer that on death of yeast cells there is a large decrease in the size of the cells and a marked decrease in their specific resistance.

DISCUSSION

In the foregoing we have attempted to analyze a system of resistances as used by several investigators for the measurement of electrical conductivity into its component parts and to determine by direct calculation the specific resistance of the suspended cells. In order to bring such a system as is represented by an inhomogeneous suspension amenable to treatment by such simple formulas as our derivations have produced, it has been necessary to make such simplifying assumptions as have been indicated above. The question of course arises as to the validity of such theoretical treatment and to what extent the expressions arrived at give correct values. The specific resistance of cells is in itself an unknown quantity, as no one has offered any measured values up to this time. However, our expressions lend themselves to direct testing by the calculation of the cross-section "a" and comparison of this value with the fractional volume occupied by the cells which may be determined fairly closely by direct means. While we consider that further testing of our expressions are necessary, nevertheless the close agreement of these two values in our preliminary experiments here reported indicate that the formulae give values which are approximately correct. Still further evidence demonstrating the validity of our derived expressions is given by our calculations of a decrease in the cross-sectional area occupied by bacterial and yeast cells on death. This corresponds to the findings of Green and Larson that B. coli decreases in size on death, which finding was obtained by direct measurement. We therefore have sufficient evidence to warrant the above theoretical treatment of an inhomogeneous system and may believe that our values found for the resistivity of bacterial and yeast cells are

approximately correct. It is to be noted that the values for the specific resistance of B. coli found in the two experiments subjected to our calculations are of the same order of magnitude, but differ by several hundred ohms. Whether this is due to experimental error which in this type of experimentation is necessarily large, or whether the resistivity is dependent on some factor such as the variation in the electrolyte content of the cell, must be determined by further work.

Our calculations show that the conductance of B. coli is not increased by a thermal death and even further indicate that the resistivity is increased. This corresponds to the findings of Green and Larson and also to those of Brooks that B. coli dead does offer considerable resistance. This is greatly at variance with the conclusions of Shearer and the generalizations of Osterhout.

Our calculations on the resistivity of yeast cells are noteworthy. The specific resistance of these cells, when alive, is much higher than B. coli, but after death the resistivity drops to a low value indicating a greatly increased conductance on death. This indicates that observations made on one kind of cell by electrical conductivity measurements cannot be used safely for extensive generalizations. The properties of electrical conductance of any type of cell and changes in this conductance seem rather to represent structure and processes peculiar to that cell species.

SUMMARY

Theoretical consideration is given to an inhomogeneous suspension as a system of resistances.

The derivation of formula is presented leading to expressions by means of which the specific resistances of suspended cells and their fractional volume of the suspension may be calculated.

The specific resistance of live B. coli is found to be about 1000 ohms and the specific resistance of S. cerevisiae about 4,000 ohms.

After death by heat the specific resistance of B. coli is increased.

After death by heat the specific resistance of S. cerevisiae is decreased.

On death by heating the volume of bacterial and yeast cells as determined by calculation decreases.

STUDIES OF FUSIFORM BACILLI AND SPIROCHETES

VII. FUSIFORM BACILLI AND SPIROCHETES IN RELATION TO PHARYNGOMYCOSIS

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Two views have been held with reference to the etiology of pharyngomycosis. The one originated by B. Frankel [1] in 1873 assumes that pharyngomycosis is a definite primary infection caused by a fungoid organism, usually termed Leptothrix. The other assumes that the primary factor is some alteration in the epithelium of these parts of such a character as to permit or favor the development of the fungus organisms which commonly inhabit the buccal mucosa, especially the leptothrix. The latter view was elaborated first by Siebenmann [2] in 1895 and later by Colwell,[3] Wood [4] and others.

Colwell in his historical review of the condition states that Siebenmann showed that the more important histologic condition was not the presence of the leptothrix so much as the hornified condition of the epithelium. He states that the importance of the leptothrix as a causative factor is much in doubt. The leptothrix thrives on necrotic tissue, as in dental caries, and the decaying horny epithelium forms a suitable soil.

Wood believes that the etiology of hyperkeratosis of the tonsils is a decreased functional activity of the tonsil, while a portion of the cryptal epithelium still possesses its physiologic tendencies toward growth. He places particular emphasis on the importance of dissociating the leptothrix or any other micro-organism from an etiologic relationship with lacunar hyperkeratosis. He speaks of this condition as a pure "vagary of function" on the part of the epithelium of the tonsillar lacunae.

Some years ago one of us called attention to the nature and structure of granules which commonly appear in the crypts of tonsils.[5] It was shown that they have a raylike structure simulating actinomyces with which they have been confused, but to which they have no relation whatsoever. They are usually composed of 4 elements: a profuse growth of an unbranched, threadlike organism, among which are interspersed many fusiform bacilli and spirochetes, and also coccal forms, chiefly streptococci. The coarser threads may be a stage in the development of the fusiform bacilli, since it is known that the latter may grow in long forms simulating those seen in the granules. The granules as made up of these 4 different forms are firm, gray or yellow bodies,

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[1] Berl. Klin. Wchnschr., 1873, 10, p. 94.
[2] Arch. f. Laryngol., 1895, 2, p. 365.
[3] Illinois Med. Jour., 1903, 5, p. 480.
[4] Tr. Am. Laryng. Assn., 1906, 28, p. 194.
[5] Davis, D. J.: Jour. Infect. Dis., 1914, 14, p. 144.

from one to several mm. in diameter, and are very foul smelling. They are found usually in the bottom of the crypts, may be multiple and from time to time are ejected by coughing or by pressure on the tonsils. On the teeth also appear structures exactly comparable to the tonsil granule. Normally just above the gingival margin a mass of bacteria grows which in structure and arrangement of elements and in smear preparations is quite indistniguishable from the tonsil granule.[6]

Since recognizing the structure of the crypt granules, it has been a matter of interest whether definite pathologic changes might be brought about by the development of these organisms either within or outside the tonsils. In tonsils containing these granules one sees no noteworthy changes within the crypts adjacent to the granules. Occasionally, however, the epithelium may be thickened with some tendency toward hyalinization. Whether these changes have anything to do with the presence of the granules is not known, but it is probably doubtful since they frequently occur in their absence. It was on this account that we became interested in pharyngomycosis from this point of view, since here we have to do with patchy lesions on the lingual and faucial tonsils and adjacent lymphatic structures, evidently associated with a mycotic growth.

Four cases have been studied in young adults from about 20 to 35 years of age. Their complaints were in no instance serious, and usually they were unaware of the presence of the lesions. In one case there was a hypersensitiveness causing a sensation of choking and gagging at times. This patient had had a tonsillectomy performed 6 years previously and the mycosis appeared on tonsillar remnants. Others complained of slight but persistent cough. Wood believes that this irritative cough found in some of these cases is due to pressure of the epiglottis on granules located in the lateral glosso-epiglottidean folds.

On examination of the throat there was seen usually on the tonsil and in one case on the posterior-pharyngeal wall several whitish or yellowish white patches, well defined, from two to several mm. in diameter, quite prominent, and often adjacent to the crypts. They were firm or even horny and being adherent were removed with difficulty, leaving a bleeding surface.

In 3 of the 4 cases the tonsils were removed. Smear preparations were made in all cases direct from the throat before the tonsillectomy. Sections were made of the tonsils and the results of the examination

[6] Davis, D. J., and I. Pilot: Jour. Am. Med. Assn., 1922, 79, p. 944.

were quite alike in all cases, so it is unnecessary to describe each case in detail.

It was found that the tonsils contained regions of thickened epithelium which had become hornified. Some of these were on the surface, others in the crypts. The upper layers of cells became dense and were arranged in layers which become separated and are removed with difficulty (fig. 1). On and between the superficial layers are found

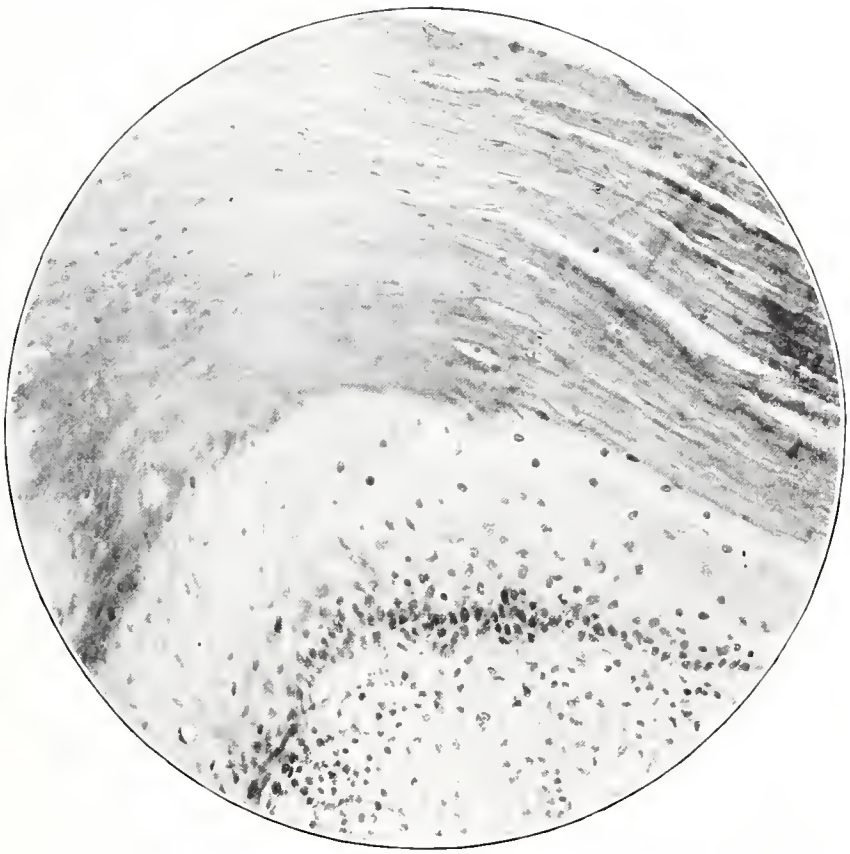

Fig. 1.—Pharyngomycosis; section of tonsil showing hyperkeratosis. Hematoxylin and eosin stain; × 250.

the growth of organisms to be described. The tissues below the epithelium and indeed the lower layers of epithelium show no alterations worthy of note.

The mycelial growth appears on and in the horny layers, growing profusely between the separate strata (fig. 2). It is for this reason apparently that the whitish patches are so adherent to the epithelium. The growth does not extend down to the living tissues.

For the minute study of the organisms preparations were studied both in hanging drops and smears stained in a variety of ways, usually with dilute carbolfuchsin, methylene blue and gram stain.

The growths were found to contain a mixture of desquamated hornified epithelial cells with many bacteria and filaments. From the lower layers grow many coarse filaments terminating at the surface and sides of the growth in brushlike structures, in every way identical

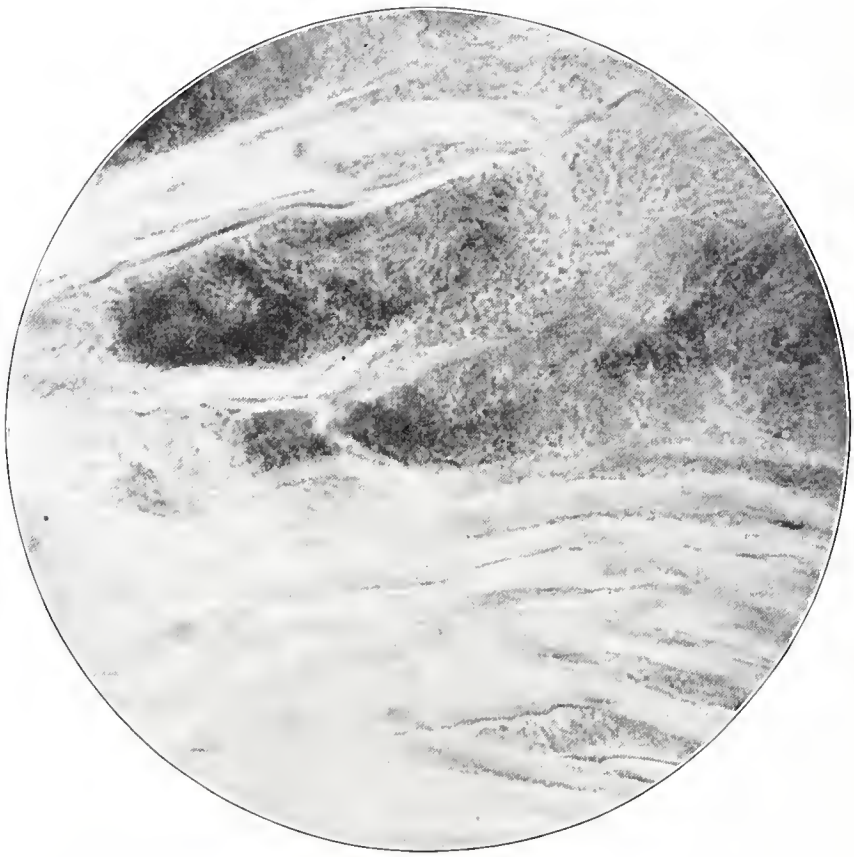

Fig. 2.—Pharyngomycosis; bacterial masses growing between hyalinized layers of tonsil epithelium; × 1,000.

with the brushlike terminals described at the margins of the tonsil and teeth granules. There may be seen a central shaft made up of coarse filaments about which are arranged more or less perpendicularly fusiform and oval bacilli, giving rise to an arrangement like a test-tube brush. Interspersed were many coarse spirochetes, in every way like those in tonsil and teeth granules, and also many gram-positive cocci, chiefly of the streptococcus type. The coarser filaments and the fusi-

form bacilli are weakly positive to Gram's stain. The structures may be made out best by placing a small amount of the growth in glycerol on a slide covered with a cover-glass pressed down carefully. The organism may be immersed in water and a small amount of stain allowed to flow under the cover-glass, thus lightly staining the organisms. By teasing carefully the growth on a slide and staining rather deeply with carbolfuchsin, the various elements in the mass may also be studied. Such a preparation is shown in fig. 3. Many fusiform bacilli are seen, also several long coarse threads, many with pointed

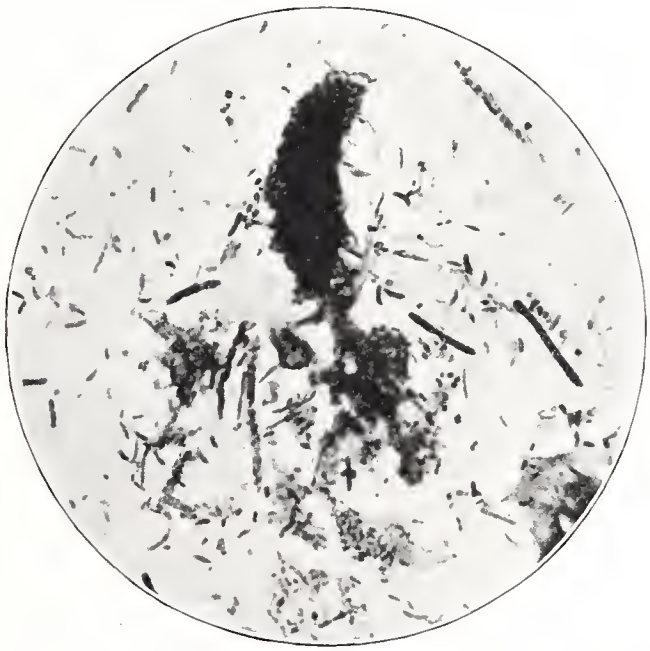

Fig. 3.—Pharyngomycosis; smear of mycotic patch showing fusiform bacilli, spirochetes, cocci and threads; also a brushlike bacterial mass; × 1,200.

ends and containing definite granules. Coarse spirochetes are seen here and there, and the coccal forms appear often in pairs, small clusters or chains. Figure 3 also shows the brushlike structure having a central curved shaft, made of the coarse filament with many cocci, oval and fusiform organisms arranged about it.

As indicated, the point arises whether the hyperkeratosis is primary or secondary to the growth of the micro-organisms. At present we think that it is primary, since there exists no reaction in the deeper layers of the mucosa. The hornified layers gradually emerge from the lower layers of the mucosa and the bacterial masses grow between the

former, but do not reach down to the latter. This no doubt explains why there are no symptoms ordinarily in this condition and why the mucosa adjacent to the patches ordinarily reveals no noteworthy change. It should be noted that a hyperkeratosis of the tonsil, especially of the crypts, is not uncommon,[2] and it is not to be understood that mycosis develops in every tonsil which shows this hornification of the epithelium, either in the crypts or on the surface.

The observation which deserves emphasis we believe is the apparent identity of this growth with tonsillar and dental granules. Evidently some factor is responsible for the hyperkeratosis; what that may be, we do not know, but upon and between the layers the organisms grow, thus becoming adherent. If this view is correct, pharyngomycosis is a manifestation of the growth of the organisms found in tonsillar crypts and on the teeth; the thickened epithelium merely furnishes a suitable medium.

The question may be raised as to the relation of this condition to Vincent's angina. The latter appears to be a definite infection with a fusiform bacillus and a spirochete. For some reason, probably because of a high virulence, they are able to gain a foothold.

It should be pointed out that this is not the only kind of mycosis of the tonsils or pharynx. Castellani, Douglas and Thomson[7] have described a large number of different mycoses, many of them no doubt of importance in tropical countries, but seemingly rare in this country.

SUMMARY

Pharyngomycosis is primarily a localized hyperkeratosis of the pharyngeal or tonsillar epithelium, the cause of which is not known. In the hyalinized epithelium grow mycotic masses of filaments (leptothrix), fusiform bacilli, spirochetes, and cocci. The structure seems identical with tonsillar and dental actinomyces-like granules.

[7] Jour. Trop. Med. & Hyg., 1923, 26, p. 19.

SPECIFIC STREPTOCOCCUS AGGLUTININS IN CONCENTRATED SCARLATINAL SERUM

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On account of the difficulty of demonstrating streptococcus agglutinins in scarlet fever serum, the effect of concentrating such serum with ammonium sulphate as described by one of us [1] has been tried.

Eight strains of hemolytic streptococci from scarlet fever (2 from throats and 6 from wounds present when the fever began) and 7 strains from other sources were used. All these strains had been tested with the serum of a rabbit immunized with a hemolytic streptococcus from the throat of a patient with an acute case of scarlet fever; the scarlatinal strains were all agglutinated in high dilutions by this serum, while the others were not agglutinated at all. These results are in accord with previous results [2] of opsonic, agglutination, absorption, and protection experiments, showing that the hemolytic streptococci usually associated with scarlet fever belong to a distinct immunologic group.

The cocci were grown for 24 hours in 1% dextrose broth mixed with ascites fluid 1 : 3; the culture was then centrifugated, and the cocci suspended in salt solution. The scarlet fever serum was concentrated by precipitation of the euglobulin and the lower pseudoglobulin fractions with a saturated solution of ammonium sulphate, in the proportion of 2 cc. of serum to 1.2 cc. of ammonium sulphate solution; after standing at room temperature 30 minutes the tube was now centrifugated, the fluid poured off, the tube inverted and flamed to precipitate the ammonium sulphate and serum still left on the sides of the tube, in order to minimize the amount of ammonium sulphate which would be mixed with solution of the precipitate. The precipitate was then dissolved in 0.4 cc. of salt solution, and this solution is designated as concentrated serum.

The concentrated and the unconcentrated serums were diluted 5 times with salt solution and an equal amount of bacterial suspension

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[1] Herrold, R. D.: Jour. Am. Med. Assn., 1923, 81, p. 203.

[2] Tunnicliff, R.: Ibid., 1920, 74, p. 1386; 75, p. 1339; Jour. Infect. Dis., 1922, 31, p. 373. Bliss: Bull. Johns Hopkins Hosp., 1920, 31, p. 173; Jour. Exper. Med., 1922, 36, p. 575. Gordon: Brit. Med. Jour., 1921, 1, p. 632.

added to each. Final dilutions of concentrated serum lower than 1 : 5 gave confusing results on account of partial coagulation of the mixture, when incubated for 3 or 4 hours at 55 C. The agglutinating mixtures as well as the control tubes with salt and ammonium sulphate solutions were incubated for 4 hours at 55 C. and read. With concentrated serum little agglutination occurred before 3 or 4 hours.

The serum from 15 scarlet fever patients, 8 convalescent diphtheria patients, and from a woman who had had scarlet fever 2 years previously, was examined. The serum from 3 scarlet fever patients was examined twice. Of the 4 scarlet fever serums collected before the 11th day of the disease, only 1 (7th day) agglutinated scarlet fever streptococci. Of the 14 specimens collected between the 18th and 28th days of the disease, 13 agglutinated scarlatinal strains. The single negative serum came from the patient whose serum agglutinated on the 7th day. One specimen agglutinated 2 scarlet fever strains but not a 3rd; another agglutinated only one of these 3 strains; the others agglutinated all the scarlatinal strains examined.

No agglutinations could be demonstrated in unconcentrated scarlet fever serum in dilutions of 1 : 10 nor in the concentrated serum in dilutions higher than 1 : 20 or 1 : 40.

As a rule, the scarlatinal strains were completely agglutinated, but with some serums the agglutination was only partial, although distinct. In only one case was the patient's streptococcus tested with the patient's serum.

Unconcentrated as well as concentrated scarlatinal serum failed to agglutinate any of the nonscarlatinal streptococcus strains. Concentrated and unconcentrated serum from convalescent diphtheria patients and from a woman who had had scarlet fever 2 years previously failed to agglutinate any of the streptococcus strains. All control tests were negative.

In a recent article, Bliss [2] concludes that the degree of agglutination in convalescent scarlet fever serum examined by him was too limited to draw any conclusions as to specificity. His table shows that the serums of 2 patients which did not agglutinate on the 4th and 6th days of the disease, agglutinated later on the 18th day. It would appear from our experiments that agglutinins for scarlatinal streptococci appear in the blood usually about the 18th day of the disease.

SUMMARY

Concentration with ammonium sulphate is useful for demonstrating specific streptococcus agglutinins in scarlatinal serum. Unconcentrated scarlet fever serum 1 : 10 fails to agglutinate hemolytic streptococci, specific for scarlet fever. When concentrated with ammonium sulphate, 25% of the scarlet fever serums collected before the 11th day of the disease and 92% of the serums collected after the 18th day, agglutinated scarlatinal strains of streptococci, but not the other streptococci tested. Serum, unconcentrated and concentrated, from diphtheria patients and from a patient who had scarlet fever 2 years previously, failed to agglutinate any of the streptococci examined.

STUDY OF CULTURAL REQUIREMENTS OF SPIROCHAETA PALLIDA

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The older literature on the cultivation of Spirochaeta pallida has been reviewed by Noguchi [1] and by Baeslack.[2] For additional historical data, the reader is referred to Sobernheim's article.[3] Since 1914, only two important contributions to this field have come to our notice; namely, that of Zinsser and his associates [4] and that of Rosenberger and Fanz.[5] These will be discussed in their appropriate places. While this work was in progress, Wassermann and Ficker [6] announced the discovery of a new culture medium. Thus far, they have not published any data.

All investigators agree that the problem is perplexing. It is one in which we must be satisfied to expect negative results. And yet, it is of the greatest importance in syphilology to devise simple and satisfactory methods of obtaining pure cultures of Spirochaeta pallida in order to establish Koch's postulates, to study the mechanism of infection, immunity and latency, to determine toxin and hemolysin production, and to test the hypothesis of neurotropic and dermatropic strains of this organism. The chemotherapeutist wants pure cultures in order to study the degree and mechanism of the spirocheticidal action of various new synthetic drugs, and the bacteriologist in order to determine the nature of the organism—protozoan or bacterial—to study its metabolism, and to differentiate it from closely related species such as Spirochaeta cunicili.[7]

It was with these thoughts in mind that we began, in June, 1921, an attempt to devise improved methods for the isolation and growth of Spirochaeta pallida in pure culture.

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[1] Jour. Exper. Med., 1911, 14, p. 99.

[2] Jour. Infect. Dis., 1913, 12, p. 55.

[3] Kolle u. Wassermann, Handbuch d. pathog. Mikroorganism, 1913, 7, p. 745.

[4] Jour. Exper. Med., 1915, 21, p. 213

[5] Researches from J. H. McFadden Laboratory of Jefferson Medical College, Philadelphia, 1919.

[6] Berl. klin.-Wchnschr., 1922, 1, p. 1101.

[7] Noguchi: Jour. Exper. Med., 1922, 35, p. 391; Kolle and Ruppert: Med. Klin., 1922, 18, p. 620; Warthin, Buffington and Wanstrom: Jour. Infect. Dis., 1923, 32, p. 316.

Noguchi,[1] in 1911, employed the spirochete-containing testicular tissue of rabbits which had been inoculated with human syphilitic material for the purpose of cultivating the spirochete. By repeated passage through rabbits, he succeeded in obtaining "spirochaeta almost free from banal bacteria." This method, although the best available at present, is too complicated for routine cultivation of spirochetes in material obtained from contaminated chancres, condylomas, etc. Unfortunately, the methods of purification, isolation and cultivation recommended by Hall,[8] Heller,[9] and Kendall[10] for anaerobic bacteria are not applicable to this organism. We have devoted much attention and time to the use of various new dyes and chemicals, such as those investigated by Churchman and his co-workers,[11] by Krumwiede,[12] and by Balls and Moral,[13] with the hope of finding one or more which would kill off the contaminating micro-organisms, without being at the same time spirocheticidal. These experiments are still in progress and will be published.

Not only is it at present extremely difficult to isolate pure cultures of Spirochaeta pallida from syphilitic rabbit material, but once having purified the strain by repeated passage through rabbits, the culture medium, described by Noguchi as the most suitable for its growth in vitro, also offers a great many obstacles and demands extraordinary care. It is no easy matter to obtain pieces of fresh, sterile rabbit kidney and fresh fibrin-forming bile-free ascitic fluid at all times. It seemed, therefore, worth while to investigate some of the physico-chemical characteristics of Noguchi's culture medium for Spirochaeta pallida in the light of the various recent researches on bacterial metabolism and of the newer anaerobic methods which have appeared since the publication of Noguchi's papers. We found that many of these factors had already been analyzed, particularly by Gates and Olitzky[14] and by Kligler and Robertson.[15] They may be summarized as follows:

1. Ascitic fluid grows progressively alkaline on exposure to the air for a long time, due to loss of CO_2. Sterile rabbit kidney undergoes autolysis with the production of acid. This acid becomes gradually

[8] Ibid., 1920, 27, p. 576.
[9] Jour. Bacteriol., 1921, 6, p. 445.
[10] Jour. Infect. Dis., 1921, 29, p. 277.
[11] Jour. Exper. Med., 1912, 16, p. 221 and p. 822; 1921, 33, p. 569.
[12] Ibid., 1914, 19, p. 501.
[13] Jour. Infect. Dis., 1918, 23, p. 382.
[14] Jour. Exper. Med., 1921, 33, p. 51.
[15] Ibid., 1922, 35, p. 303.

diffused throughout the medium and creates a zone of favorable hydrogen-ion concentration.

2. Ascitic fluid contains little buffer substances, but does perhaps furnish "vitamines" which favor the nutrition of Spirochaeta pallida. When liquified agar is mixed with it, a coagulum is formed in which the spirochetes are enmeshed, and their initial growth is favored. The proper degree of viscosity of the medium is thus also assured. H. Jones [16] has shown, especially in reference to streptococci, that "ascitic fluid" alters the tolerance of the organism to the toxic hydrogen ions. Autoclaving the ascites broth reduces it to the condition of ordinary broth in this respect.

3. Buffer and nutritive substances, and possibly "vitamines," dialyze out of the kidney as well as from the red blood cells contained therein. The piece of kidney furthermore acts as a nidus for the initial growth of young spirochetes.

4. All tissue cells contain reducing substances and thus a zone of reduced oxygen tension is created in the vicinity of the piece of kidney. This is evidenced by the reduction of methylene blue for a distance above the latter, depending on its size.

The plan of our investigation was by proper attention to these factors to build up a medium the ingredients of which could be more readily obtained and the essential characteristics of which would be more or less quantitatively controlled.

THE VIABILITY OF SPIROCHAETA PALLIDA IN MEDIUM OF VARIOUS HYDROGEN-ION CONCENTRATIONS; OPTIMUM P_H REQUIREMENTS

The importance of controlling hydrogen-ion concentration in the cultivation of various micro-organisms has been emphasized in numerous recent investigations which are well summarized in Clark's monograph.[17] The optimum P_H requirements of Spirochaeta pallida, has, to our knowledge, not yet been determined. As indicated, the final reaction of Noguchi's medium varies with the size of the piece of rabbit kidney and with the reaction of the ascitic fluid. When one attempts to determine the P_H of any given tube of this medium by the colorimetric methods of Clark, many technical difficulties are encountered. Like Hall,[18] we did not succeed in finding a suitable indicator which would

[16] Jour. Infect. Dis., 1920, 26, p. 160.
[17] Determination of Hydrogen-Ions, 1920.
[18] Jour. Infect. Dis., 1921, 29, p. 321.

not be reduced when incorporated in anaerobic culture mediums. For this and other reasons, we chose a modification of Schereschewsky's [19] gelatinized horse serum as the stock culture medium in this work. We also employed unheated horse serum broth, although we were aware that Noguchi [1] as well as Zinsser, Hopkins and Gilbert [4] had found this medium unsuitable. Our object was to test any possible effect of heating on the P_H of the medium, knowing that the CO_2 is driven off during this process. The cultures employed were those originally isolated by Noguchi and used by the H. K. Mulford Co. in the manufacture of their "Luetin."

Into a series of flasks were placed 50 c c. of neutral Huntoon's [20] "hormone" broth, and their P_H was adjusted to range from 6.6 to 8.4. Normal sterile horse serum was now added in the proportion of 1 part of serum to 4 of broth. The medium was tubed in special Pyrex "spirochete" tubes 20x1.5 cm. under sterile precautions, and its P_H tested after 30 minutes' incubation. The tubes were then divided into two series: The first was left unheated, the second was heated in a water bath at 80 C. for one-half hour. The latter medium is fluid in consistency and opaque. After prolonged incubation with or without inoculation, the proteins are coagulated and settle down to the lower half of the tube. Spirochetes can be found in abundance in the upper, clear portion as well as in the sediment. All tubes were sealed with a layer of sterile yellow petrolatum 1 cm. thick and incubated for 3 days at 37 C. before use. The P_H was now again determined and inoculation made with equal quantities of culture of Spirochaeta pallida. Capillary pipets attached to a rubber bulb were used for this purpose. We did not find it necessary to employ any anaerobic jars. Examinations were made under the dark-field microscope every week for 3 weeks.

For P_H determinations, the technic of Clark [17] was used: One c c. of culture medium was diluted with 4 c c. of double distilled neutral water, and comparisons were made in a Walpole comparator with suitable standards of methyl red and phenol red. The intensity of growth was judged by the number of spirochetes per dark field, the degree of their activity and motility, their morphology (length, thickness, integrity and number of curves) and by their general appearance. Selection from the data are shown in tables 1 and 2.

[19] Deutsch. med. Wchnschr., 1909, 35, pp. 835, 1260 and 1652; 1912, 38, p. 1335.
[20] Jour. Infect. Dis., 1918, 23, p. 168.

SUMMARY OF RESULTS

Changes in Uninoculated Culture Medium.—The variations in hydrogen-ion concentration of uninoculated culture medium were recently studied by Linwood and Highberger.[21] Kligler and Robertson [15] have emphasized the fact that during incubation the P_H of culture medium containing animal fluids under paraffin oil changes progressively

TABLE 1

VIABILITY OF SPIROCHAETA PALLIDA IN MEDIUMS OF VARIOUS PH
Series 1. Unheated horse-serum-"hormone" broth.

P_H of Original Hormone Broth	P_H of Serum Broth Just Before Planting	P_H of 7-Day Old Culture, S. pallida	Results of Examinations with Dark-Field Microscope, 7th Day	P_H of 14-Day Old Culture, S. pallida	Dark-Field Examinations, 14th Day	P_H of 21-Day Old Culture	Dark-Field Examinations, 21st Day
6.6	6.6	6.88	Negative	6.8	Negative	6.4	3 per field; M. & A. poor
6.8	6.9	6.9	Negative	6.8	10-20 per field; M. & A.* fair	6.6	10 per field; M. & A. fair
7.0	7.1	7.2	Negative	7.2	Negative	7.2	Negative
7.2	7.4	7.3	Negative	7.1	10-15 per field; M. fair A. good	6.9	10 per field; M. & A. fair
7.4	7.3	7.2	Negative	6.9	30-40 per field; M. & A. good	6.3	20-30 per field; M. & A. good
7.6	7.5	7.5	Negative	7.3	10 per field; M. & A. good	6.3	10-15 per field; M. & A. good
6.8	7.5	7.4	Negative	7.4	1-5 per field; M. & A. poor	7.0	10-15 per field; M. & A. good
8.0	7.6	7.4	10-15 per field; M. & A. very good	7.1	10-15 per field; M. & A. fair	6.3	10-15 per field; M. & A. good
8.2	7.6	7.5	Negative	7.5	Negative	7.1	10-15 per field; M. & A. very good
8.4	7.7	7.2	10-20 per field; M. & A. good	6.9	10-15 per field; M. & A. good	6.3	10-15 per field; M. & A. fair

* M. & A. = morphology and activity.

toward alkalinity, irrespective of the initial reaction. This, no doubt, is due to loss of CO_2. In the present study, the diffusion of CO_2 was prevented by the use of the petrolatum seal, the advantages of which are discussed by Gates and Olitzky [14] and by Hall.[22]

(The amphoteric action of serum proteins is well seen in column 2 of table 1.) The tubes from P_H 6.6-7.4 suffered an increase, while

[21] Ibid., 1920, 26, p. 457.
[22] Jour. Bacteriol., 1921, 6, p. 1.

those from 7.6 to 8.4 suffered a decrease in alkalinity on the addition of serum to "hormone" broth. But these changes were not permanent, for after 2 days' incubation under a petrolatum seal, all tubes of the unheated series became more acid, owing probably in part to autodigestion of the serum proteins. In the heated series, on the other hand, the enzymes having been destroyed, the P_H changes after the addition of serum were permanent and showed practically no change even up to 42 days' incubation under vaselin seal.

The Viability of Spirochaeta Pallida in Unheated Horse Serum Broth of Various P_H.—As seen in table 1, growth is possible in unheated horse serum broth under a petrolatum seal, but only when the reaction has been properly adjusted and stabilized at about P_H 7.6. In more acid medium, up to P_H 6.9 growth is also possible, but is retarded. It will also be noticed that the P_H of the medium progressively decreased, no doubt owing to acid production by the spirochetes.

Viability of Spirochaeta Pallida in Heated Serum Broth of Various P_H.—The results of these experiments are shown in table 2. The optimum growth on the 7th day was obtained when the reaction at the time of planting had reached P_H 7.5 to 7.9. The spirochetes, however, remained viable up to P_H 6.8. In 3 weeks old cultures, the best growth was noted when the original P_H was about 7.8. That the Spirochaeta pallida produces acid in this medium is quite evident when we observe the fairly uniform and progressive fall in P_H in planted tubes as compared with the constant P_H of the unplanted controls. By the 42nd day, all tubes had reached a reaction of about P_H 6.1, and the spirochetes had degenerated in morphology and activity.

Anaerobiasis, Reducing and Absorbing Substances.—The exact degree of anaerobiasis (oxygen tension [?]) required for the growth of Spirochaeta pallida is not known. Nor have we the proper chemical criteria for judging this. Ivan C. Hall [22] and Gates and Olitzky [14] have accepted the reduction of methylene blue as an index of a strictly anaerobic condition. The recent electro-metric researches of Clark and Cohen [23] on oxidation-reduction equilibria have, however, thrown a new light on this subject. They have shown that the reduction of methylene blue indicates only that "a certain definite level of reducing intensity has been reached and that other indicators such as indigo carmine or indophenol reveal only that the solution has reached that narrow zone of reduction potential or reducing intensity characteristic of the given

[23] Science, 1921, 54, p. 557; Clark: Jour. Washington Acad. Sc., 1920, 10, p. 255.

dye." They also emphasize the fact that the hydrogen ion concentration affects the reduction potential of the various dyes. Our own experiments have shown that a number of substances credited with reducing power actually adsorb methylene blue. We observed the precaution to determine whether the dye would regain its color on

TABLE 2

VIABILITY OF SPIROCHAETA PALLIDA IN MEDIUMS OF VARIOUS PH

Series 2. Horse-serum "Hormone" Broth Heated at 80 for One-Half Hour

P_H of Original Hormone Broth	P_H of Serum Broth Before Planting	P_H of 7-Day Old Culture	Dark-Field Examination, 7th Day	P_H of 14-Day Old Culture	Dark-Field Examination, 14th Day	P_H of 21-Day Old Culture	Dark-Field Examination, 21st Day
6.6	6.9	6.88	5-10 per field; M. & A.* fair; irregular curves	6.2	10 per field; M. & A. poor	6.2	5-8 per field; M. & A. poor
6.8	7.2	6.8	5-10 per field; M. & A. fair	6.3	10 per field; M. & A. fair	6.3	10-20 per field; M. & A. fair
7.0	7.3	7.1	5-10 per field; M. & A. fair	6.88	10 per field; M. & A. fair	6.9	10-20 per field; M. & A. fair
7.2	7.4	7.25	10-15 per field; M. & A. fair	6.9	10 per field; M. & A. fair	7.1	10-15 per field; M. & A. fair
7.4	7.5	7.1	20-30 per field; M. & A. good	6.88	10 per field; M. & A. fair	6.9	10-15 per field; M. & A. fair
7.6	7.6	7.2	20-30 per field; M. & A. very good	7.0	10 per field; M. & A. fair	7.1	10-15 per field; M. & A. fair
7.8	7.7	7.3	20-30 per field; M. & A. very good	6.9	10 per field; M. & A. fair	7.1	10-15 per field; M. & A. fair
8.0	7.9	7.4	20 per field; M. & A. good	6.9	15 per field; M. & A. good	7.3	10-15 per field; M. & A. fair
8.2	7.7	7.4	20 per field; M. & A. good	6.9	20 per field; M. & A. good	7.0	40-50 per field; M., good; A., fair
8.4	7.9	7.45	30-40 per field; M. & A. excellent; best of all	7.1	Excellent growth, 30-40 per field	7.0	10-15 per field; M. & A. fair

* M. & A. = morphology and activity.

exposure to the air after having seemingly been reduced to its leukobase. We also buffered our solutions at a constant P_H of 7.4.

Douglas, Fleming and Colebrook [24] succeeded in growing anaerobic organisms, such as B. refringens and B. tetani under apparently aerobic conditions by placing at the bottom of the culture tubes various substances which possess adsorbing powers. In the opinion of these

[24] Lancet, 1917, 2, p. 530.

authors, the bacilli lodge and multiply in the meshes created by these substances, since anaerobic conditions are thus created locally. We attempted to apply their results to the present investigation. Using as the basic medium Huntoon's [20] "hormone" broth containing 0.1% agar and 0.2% sodium phosphate, P_H 7.6 to 7.8, we incorporated the following sterilized substances which were placed at the bottom of the tubes, and then seeded with Spirochaeta pallida: gauze and filter paper (in small pieces), capillary tubules, washed asbestos wool, charcoal, Kieselguhr bread crumbs and bran. The results were negative.

TABLE 3

THE GROWTH OF SPIROCHAETA PALLIDA ON VARIOUS HEATED TISSUE MEDIUMS AND IN FRESHLY PREPEARED HUMAN LEUKOCYTIC EMULSIONS

Name of Medium	Note on Method of Preparation	Summary of Results of Dark-Field Examinations
Hall's sheep's brain medium	$CaCo_3$ at bottom of tubes; P_H when planted 7.6	2-5 spirochetes per field, morphology and activity fair; tubes slightly darkened
Broth from external genitalia of sheep (male and female)	Prepared after technic of Huntoon;[20] formed viscous broth; diluted with broth 1:4	3-10 per field, morphology and activity fair
Testicular infusion agar.....	P_H adjusted to 7.6 technic of Clark	10-30 per field, morphology and activity fair
Testicular infusion.....	P_H adjusted to 7.6 technic of Erickson and Albert	Negative
Trypsinized beef heart.....	Technic of Stickel and Meyer[42] 0.2% Na_2HPO_4	Poor
Cooked meat medium.....	Technic of Holman +0.2% Na_2HPO_4 1% peptone P_H 7.6-7.8	Very poor
Leukocytes emulsion (from human blood) added to 0.1% hormone agar	Blood collected in 1% sodium citrate in 0.85% NaCl solution; leukocytes removed after centrifugalization	2-3 per field; good morphology and activity

Hence we turned our attention to various reducing substances and catalysts, such as platinized asbestos and palladized asbestos which play so important a rôle in Wieland's experiments on dehydrogenation,[25] and finally to chrysarobin and neorobin.[26]

Platinized asbestos when scattered throughout the medium gave surprisingly good growth from 5-20 spirochetes per dark field of good morphology and activity.

"Vitamines." (Growth Accessory Substances) in the Cultivation of Spirochaeta pallida.—During the past few years, these phases of bac-

[25] Quoted by Dakin: Physiol. Rev., 1921, 1, p. 394.
[26] Schamberg, Raiziss and Kolmer: Jour. Cutan. Dis., 1915, 33, p. 98.
[27] Jour. Infect. Dis., 1921, 29, p. 171.
[28] Jour. Exper. Med., 1919, 30, p. 31.

terial nutrition have been intensively investigated by Davis,[27] Kligler,[28] Lloyd,[29] McLeod and Wyon,[30] Rivers,[31] and others. More recently Thjötta and Avery [32] have found that hemophilic bacilli require for their growth two substances present in the blood—a vitamine-like substance and an X substance. These two substances occur also in various raw plant tissues, especially in the potato. The vitamine-like principle in blood is destroyed at 120 C. in the autoclave; the X principle is not. Both are unimpaired in boiling water for 10 minutes. It is possible, in our opinion, that these substances are related to "gluta-thione," an auto-oxidizable substance present in all living cells, recently discovered by Hopkins.[33]

In applying these principles to the cultivation of Spirochaeta pallida, our basic medium was Huntoon's "hormone" broth prepared without filtering through paper, and hence without removing certain of the accessory substances of nutrition which are usually lost in the older methods of preparing mediums employed by Noguchi. This medium alone, or containing small amounts of agar, as suggested by Hitchens,[34] was inadequate. The various yeast mediums were then tested, especially those of Kligler [35] and of Eberson,[36] with numerous modifications. These mediums, as well as combinations of Huntoon-Hitchens hormone agar enriched by the addition of yeast extract prepared after the method of Thjötta and Avery,[32] and of neutralized orange juice were similarly negative.

Finally, we began to use fresh sterile raw potato as a source of accessory and reducing substances. In preliminary experiments, we found no difficulty in demonstrating the reduction of methylene blue, using small sterile pieces of potato of equal size placed in test tubes under a petrolatum seal and buffered with Acree's universal buffer [37] solution adjusted to P_H 7.5. These uniform sized pieces of sterile raw potato were obtained by a special simple device consisting of a piece of glass tubing rounded off at one end but not at the other. A piece of solid glass rod was passed through the tube and served as a "piston." The potato was washed, scrubbed and boiled for half a minute in water

[29] Jour. Path. & Bacteriol., 1916-1917, 21, p. 113.
[30] Ibid., 1921, 24, p. 205.
[31] Jour. Am. Med. Assn., 1921, 76, p. 1744.
[32] Jour. Exper. Med., 1921, 34, pp. 97 and 455.
[33] Biochem.-Jour., 1921, 15, p. 286.
[34] Jour. Infect. Dis., 1921, 29, p. 390.
[35] Jour. Bacteriol., 1919, 4, p. 183.
[36] Jour. Am. Med. Assn., 1919, 72, p. 852.
[37] Jour. Infect. Dis., 1921, 29, p. 7.

and sterilized with alcohol and then flamed. It was then cut almost in half with a sterile knife and fork; by means of the sterilized piston and tube, cylindrical pieces of potato were bored out and plunged directly into spirochete tubes without any additional exposure. In order to test the viability of Spirochaeta pallida in a simple serum-free medium with potato as the source of vitamine and reducing substances, we first had to neutralize the natural acid reaction of the potato (P_H 6-7). After several preliminary experiments, the technic adopted was as follows: Small pieces of sterile raw potato of uniform size were transferred to spirochete tubes containing 8 c c. of "universal buffer solution" adjusted to various reactions. These were sealed with yellow petrolatum and incubated. After 72 hours, buffer and petrolatum were removed and sterile hormone broth P_H 7.8 was poured into the tubes; the latter were again sealed and reincubated to test for sterility; 36 hours later, these tubes were inoculated with Spirochaeta pallida and dark-field examinations were made on the 7th day.

Results.—Tubes of hormone broth containing sterile, raw potato buffered at P_H 8.6-8.0 showed 3-8 spirochetes of fairly good morphology and activity. Similar or slightly better growth was obtained with a medium containing 0.5% agar and potato. All the controls were, of course, negative. Hence, sterile raw potato furnishes substances that help to sustain the life of Spirochaeta pallida.

THE APPLICATION OF VARIOUS MEDIUMS TO THE CULTIVATION OF SPIROCHAETA PALLIDA

A number of culture mediums of other authors, as well as a few of our own composition, were selected with the hope of finding one or more which would be less complex and more readily controlled than Noguchi's kidney ascites agar. Some of these mediums had been found successful, especially during the war, in the cultivation of various anaerobic bacteria. Others were chosen because it was thought that Spirochaeta pallida might have characteristics in common with the organism for which the medium was recommended. The following summary will serve as a guide to some of the types of mediums tested.

EGG MEDIUM

1. Besredka's egg broth[38]
2. Dorsett's egg slants
3. Authors' whole egg broth

[38] Ann. de l'Inst. Pasteur, 1921, 35, p. 291; 1913, 27, p. 1009.

4. Stitt's egg broth[39]
5. Hormone broth and whole hen's egg in various combination
6. Egg white and egg yolk solutions
7. Zinsser and Gilbert's[4] egg-serum medium
8. Rettger's[40] egg-meat medium
9. McCoy and Chapin's[41] coagulated hen's egg yolk.

Our whole egg broth is prepared as follows: The contents of 2 fresh eggs are removed with sterile precautions and emulsified in 200 c c. of a solution containing 1% peptone (Difco), 0.2% sodium phosphate and 0.2% agar. The P_H is adjusted to 7.8, and the medium is then slowly brought to a boil, stirring frequently. It is tubed in spirochete tubes and sterilized in the Arnold (½ hour) and sealed with 1 cm. of sterile yellow petrolatum while the tubes are still hot.

Blood Medium

10. Sheep's blood clot in various combinations
11. Trypsinized sheep's blood clot agar, prepared according to the technic of Stickel and Meyer[42]
12. Rosenberger's[5] medium with a number of modifications
13-15. Kligler's[43] medium with a few changes
16. Modifications of Young and Van Sant's[44] medium (Used defibrinated horse blood)

Serum Medium

17-18. Hormone broth containing various percentages of horse serum, heated and unheated
19-21. Hitchen's[34] 0.2% agar-hormone broth containing varying percentages of horse serum, heated and unheated
22. Horse serum (10%) in 1% hormone agar, modification of Muhlen's and Hoffmann's medium[39]
23. Authors' modification of N.N.N. medium[39] containing no meat extract or peptone, only 0.2% agar and 0.2% sodium phosphate, P_H 7.8, (autoclaved) and to which various amounts of horse serum (1 to 10%) are added. Medium is heated in Arnold sterilizer for one-half hour; some protozoa prefer a medium free from meat extract and peptone

Heated Tissue Medium; Leukocytic Emulsion Agar

24. Hall's[8] sheep brain medium.
25. Authors' medium of male and female external genital organs of sheep
26. Clarke's[45] testicular infusion agar
27. Erickson and Albert's[46] testicular infusion agar
28. Beef-heart, trypsinized after technic of Stickel and Meyer[42]
29. Holman's[47] cooked meat medium (modified)
30. Emulsion of human leukocytes added to 0.1% hormone agar

[39] Practical Bacteriology, 1920.
[40] Jour. Biol. Chem., 1906, 2, p. 71; 1908, 4, p. 45.
[41] Public Health Rep., 1922, 37, p. 102.
[42] Jour. Infect. Dis., 1918, 23, p. 68.
[43] Jour. Exper. Path., 1922, 3, p. 4.
[44] Proc. Soc. Exper. Biol. & Med., 1922, 19, p. 299.
[45] Jour. Bacteriol., 1920, 5, p. 99.
[46] Jour. Infect. Dis., 1922, 30, p. 268.
[47] Jour. Bacteriol., 1919, 4, p. 149.

SUMMARY OF RESULTS

Egg Mediums.—Of the various types of serum-free egg mediums tested, the following proved the most successful in our hands: whole egg added to hormone broth [14] heated for one-half hour in the Arnold sterilizer, and the author's whole egg broth. On one occasion, we obtained excellent growth in Besredka's egg broth, but could not duplicate this result although we tried at least 8 times. The Dorsett's egg and serum medium recommended by Zinsser and Gilbert [4] gave good growth. Since we desired to develop a serum-free medium, we tried Besredka's broth in place of serum in this medium. The modified medium did not give as good a growth. Numerous other combinations of egg-yolk and egg-white mediums gave negative results. There are probably certain constituents in the yolk as well as in the white of an egg which furnish the proper nutrition, for Spirochaeta pallida. Zinsser and Hopkins [48] suspected cholesterol, but their experiments proved unsuccessful.

Blood Mediums.—Many of the older workers on Spirochaeta pallida employed blood medium. Thus Martoni and Hoffman [3] used broth enriched with defibrinated blood of cattle, and obtained excellent growth. Rosenberger and Fanz [5] have recently revived these methods and recommend the use of freshly defibrinated rabbit blood medium "in partial vacuo" created by pyrogallol and a suction pump. They emphasize the reducing (oxygen absorbing) capacity of the erythrocytes as an important factor in this medium. Since it is often difficult to obtain fresh sterile blood in sufficient quantity, we sought to develop mediums involving the use of heated blood made from clotted blood obtained in the slaughter house. Our results were disappointing.

Of the various whole blood mediums tested, the best growth was obtained with a modification of Kligler's medium. It is significant that the addition of 0.2% agar improves this medium remarkably. This is probably due, as suggested by Hitchens,[34] to the fact that in medium containing small percentages of agar "the gel, composed of colloidal particles in a state of equilibrium, resists the penetration of oxygen and consequently offers excellent conditions for the development of anaerobic bacteria."

It is also interesting to note that the various mediums recommended by Stitt [39] for the cultivation of protozoan parasites, did not prove satisfactory for Spirochaeta pallida in the course of these experiments.

[48] Personal communication.

Serum Mediums.—Baeslack,[2] Zinsser, Hopkins and Gilbert,[4] as well as many of the European workers cited by Sobernhein,[3] prefer to use some form of serum medium similar to Schereschewsky's gelatinized horse serum.[19] Most authors emphasize the necessity of heating the serum. Thus Zinsser states "we were unable to grow the culture on horse serum without tissue, unless the serum had been heated and gelatinized." It will be noticed in referring to table 1 that the P_H of the medium (which these earlier workers did not control) plays an important rôle, and that good growth can be obtained if the broth is previously adjusted to P_H 7.6 or 7.7. Furthermore, the older workers employed the paraffin oil seal, which allows the escape of Co_2 and the absorption of oxygen. Moreover, the addition of small quantities of agar (0.2%) to unheated horse serum furnishes a good pabulum for the growth of Spirochaeta pallida, and the incorporation of 0.2% agar in a medium of heated horse serum greatly improves it. Hence, the physical characteristics of the medium are important considerations.

Heated Tissue Mediums (and Leukocytic Emulsion Agar).—A number of heated tissue mediums have been developed and especially recommended for the growth of anaerobic bacteria during the war. Zinsser, Hopkins and Gilbert [4] succeeded in growing nonpathogenic pallida on a similar pabulum. It was our aim to improve some of these and to apply them to the cultivation of Spirochaeta pallida. The male and female external genital organs of sheep were also included, since it was thought that these tissues might possess some substances favorable to the growth of spirochetes. Many testicular mediums (recommended for the gonococcus) were also tested; of the latter, the technic of Clark [45] proved the most successful. We also obtained growth on a medium of hormone agar enriched by a few drops of an emulsion of human leukocytes. Numerous other types of heated tissue medium proved of no value.

It is evident therefore that Spirochaeta pallida, even after years of cultivation on artificial culture medium, maintains its fastidious requirements, and none but carefully adjusted, controlled and stabilized mediums will be suitable for its growth. While this manuscript was being revised, our attention was called to the work of Gates [49] who succeeded in cultivating nonpathogenic spirochetes on the surface of blood-agar plates in a Brown anaerobic jar.

[49] Jour. Exper. Med., 1923, 37, p. 311.

SUMMARY

We have reviewed briefly the recently developed facts and theories concerning the physicochemical factors involved in Noguchi's medium for Spirochaeta pallida: hydrogen-ion concentration, buffer and vitamine content, anaerobiosis, presence of reducing and absorbing substances, viscocity, etc. We set out to develop additional and more definite data on these subjects and to apply this information to the elaboration of a new culture medium, the ingredients of which could be obtainable with less difficulty and the essential characteristics more quantitatively controlled than in Noguchi's ascites—kidney-agar. The results of our experiments may be summarized as follows:

The Relation of Hydrogen-Ion Concentration of the Medium to the Growth and Viability of Spirochaeta pallida.—Because of technical difficulties, we employed a modification of Schereschewsky's gelatinized horse serum instead of the original Noguchi medium in this study. We observed that:

A medium of heated gelatinized horse serum and broth remains of constant P_H when incubated under a petrolatum seal, whereas when unheated horse serum is used, the reaction changes, due probably to the activity of the enzymes.

Spirochaeta pallida can be cultivated on unheated horse serum broth only when the reaction has been adjusted to the proper range of P_H. Previous investigators who obtained negative results have overlooked this precaution.

For optimum growth in a medium of heated horse serum (prepared as described in the text), the initial reaction should be P_H 7.5-7.9. If the initial reaction of the medium is more acid than 6.9 growth is impossible.

The morphology and activity of the organism is influenced by the reaction of the medium. When the latter reaches a P_H of about 6.1 to 6.4, spirochetes are found in a state of disintegration; they lose their curves, become elongated, swollen and immobile.

Spirochaeta pallida produces acid in serum mediums.

Importance of Reducing and Adsorbing Substances in Mediums for Cultivation of Spirochaeta pallida.—The addition of adsorbing substances such as those recommended by Douglas, Fleming and Colebrook (asbestos wool, charcoal, gauze, etc.) does not make possible the growth of Spirochaeta pallida in simple mediums.

Platinized asbestos when scattered throughout a tube of 0.2% agar in "hormone" broth renders the medium favorable for the growth of Spirochaeta pallida. We were also able to cultivate the organism with the use of sterile raw potato in a specially buffered broth.

Development of New and Modified Mediums.—More than 40 available mediums of other authors (as well as a few of own composition) were selected and modified to conform with some of the physicochemical requirements possessed by Noguchi's medium.

The following gave good growth at optimum reaction—P_H 7.6-7.8 and under a petrolatum seal:

Whole egg added to "hormone" broth (1:4) heated ½ hour in an Arnold sterilizer.

Authors' whole egg broth (see text).

Modified Kligler's medium (using freshly obtained rabbit's blood).

Unheated and heated horse serum broth. These mediums are greatly improved by the incorporation of small quantities of agar (0.2%).

Most of the mediums recently developed for the cultivation of various anaerobic bacteria proved valueless for Spirochaeta pallida. Clark's testicular infusion, as well as the writer's medium, made by the addition of fresh human leukocytes to 0.1% "hormone" agar gave encouraging results.

A BIOLOGIC STUDY OF ACIDURIC BACTERIA

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The aciduric group of bacteria consists of those micro-organisms that are able to live and multiply in degrees of acidity unfavorable for the development of most microbes. These bacteria are widely distributed in nature, being found in the intestinal tracts of many animals, in milk, silage and other fermentable materials. Detailed information in regard to their biologic properties and their significance is relatively meager, however, due largely to the fact that they do not grow well on many laboratory mediums. Consequently, there has been much confusion in the past in separating different types, and not until recent years have adequate cultural tests for their differentiation been made. In view of these facts, it seemed desirable to make a somewhat systematic biologic survey of certain of these bacteria, with particular attention to their fermentative properties.

The principal types of aciduric bacilli found in the animal body are B. acidophilus (Moro [1] and Finkelstein [2]), B. bifidus (Tissier [3]), and B. acidophil-aerogenes (Torrey and Rahe [4]). Moro originally believed that B. acidophilus was an acid lover, hence the name. Rodella,[5] however, showed that many of these organisms grow well in an alkaline medium, and are more acid resisting than acid loving. Later, Kendall [6] proposed the term aciduric, meaning organisms capable of enduring considerable degrees of acidity, and most workers in this field have adopted this term as best expressing the main characteristics of the group.

The activities of these organisms are particularly interesting and important as certain of them are normal inhabitants of the human intestinal tract, and in infants are the predominant bacterial types in the lower bowel. Being acidogenic with slight proteolytic powers, they are apparently beneficial in their activities, and probably are desirable

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[1] Wien. klin. Wchnschr., 1900, 13, p. 114.
[2] Deutsch. med. Wchnschr., 1900, 26, p. 263.
[3] These de Paris, 1900.
[4] Jour. Infect. Dis., 1915, 17, p. 437.
[5] Centralbl. f. Bakteriol., 1901, 29, p. 717.
[6] Jour. Med. Res., 1910, 22, p. 153.

intestinal inhabitants. On the other hand, the usual types of bacteria found in the lower intestinal tract of adults are predominantly proteolytic, and the "autointoxication theory" rests on the more or less complete evidence of their harmful effects. Evidence is steadily accumulating which shows the advisability, in many abnormal conditions in man, of encouraging the development of these aciduric types in the digestive tract, thereby diminishing intestinal proteolysis with its resultant ill effects.

Confusion has arisen in efforts to transform the intestinal flora, largely because of the meager data concerning bacteriologic varieties within the aciduric group. The world-wide use of bulgarian milk resulted from the mistaken notion that B. bulgaricus is a normal intestinal inhabitant, and hence can be implanted in the human digestive tract. This fundamental error was due to the confusion of this organism with B. acidophilus. Within recent years, Rahe [7] has shown that these two organisms can be easily differentiated by cultural means, and thus was able to prove that B. acidophilus, and not B. bulgaricus, is normally found in the human intestinal tract.

The renewed interest at the present time in the significance of the intestinal bacteria has brought out many new facts, particularly with reference to the transformation of the intestinal flora by diet, but little attention has been paid to the detailed systematic relationships of the members of the aciduric group. The present study is concerned with such, and deals with the cultural characteristics of 64 strains of B. acidophilus and 34 strains of B. acidophil-aerogenes, isolated from 93 different sources, mainly from the sputum and feces of normal human adults. A few strains have also been secured from the intestinal tracts of the white rat, rabbit, dog, quail and from unpasteurized milk.

MEDIUMS

The aciduric bacteria grow with difficulty on the ordinary laboratory mediums, but will grow luxuriantly if unneutralized meat infusion broth containing a fermentable carbohydrate is used. Rahe [8] showed this definitely, and by using such a broth made "sugar-free," was able to demonstrate their fermentative properties. In 1919, Ayers and Rupp [9] showed the usefulness of autolyzed yeast broth as a basis for fermentation tests in studying certain streptococci which would not grow in meat

[7] Jour. Infect. Dis., 1914, 15, p. 141.
[8] Jour. Bacteriol., 1918, 3, p. 407.
[9] Ibid., 1920, 5, p. 89.

extract broth or in "sugar-free" infusion broth. Vansteenberge [10] had previously shown that yeast is readily autolyzed and that an extract from such is a good medium for the growth of yeast and lactic acid bacteria. He demonstrated that during autolysis the nitrogenous material is proteolyzed, with the formation of peptones, amino acids and other products. At the same time the hydrocarbon material is changed into dextrose and then into carbon dioxide and alcohol. He states that an extract from autolyzed yeast contains much more nitrogenous material than a similar extract from fresh yeast, but is practically free from fermentable substances. In view of these facts, I have used autolyzed yeast broth as a basis for most of the mediums employed in this study. The dried autolyzed yeast was obtained through the courtesy of the Fleischmann Company. The ease with which this medium is prepared makes it more convenient to use than unneutralized "sugar-free" infusion broth. The broth is made as follows:

Tap water	1,000 c c.
Peptone (Armour's)	10 gm.
Dried autolyzed yeast	10 gm.
NaCl	5 gm.

This mixture is boiled for 15 minutes, then flasked and autoclaved. When the broth is cold, it is shaken well and filtered through filter paper. The first part of the filtrate is rather turbid, but the remaining portion is water clear. For fermentation tests, the carbohyrate is added in 1% amounts, and the medium is steamed in the Arnold autoclave for 20 minutes on 3 consecutive days. I have tried many times to get aciduric organisms to grow in this broth in the absence of a carbohydrate, but always with negative results. B. coli will grow readily, but 12 to 24 hours' growth does not appreciably affect the hydrogen-ion concentration of the medium, thus showing the practical absence of fermentable material. The aciduric bacteria will grow in this broth only in the presence of a fermentable carbohydrate, but under such condition their growth in 18 to 24 hours is luxuriant and the P_H becomes less than 5. Consequently, the following standard has been adopted in determining the fermentation of any carbohydrate: within 5 days at 37 C. a P_H of less than 5 must be attained. This is easily determined by adding a few drops of methyl red to the culture, the cherry red color indicating the pronounced acidity of the medium.

[10] Ann. de l'Inst. Pasteur, 1917, 31, p. 601.

ISOLATION

Material to be examined was inoculated into acetic acid dextrose unneutralized infusion broth, prepared by adding 0.25% glacial acetic acid to the broth. The resulting P_H was 4.8 to 5.0. After 3 successive transfers at 24-hour intervals, a plating was made on whey agar or dextrose yeast agar. When the colonies were well developed on the plates, isolated ones were picked to tubes of unneutralized glucose yeast broth. After 24 hours Gram stains were made, and if gram-positive bacilli were present in apparently pure culture, transplants were made to whey agar and carried as stock cultures.

The dehydrated whey broth (Difco) has been a great convenience for stock cultures, whey broth containing 1.3% agar being used for this purpose throughout the work.

MORPHOLOGY

The organisms of the aciduric group are generally considered to be gram-positive rods, nonmotile, somewhat pleomorphic, and varying in length from 1 to 11 microns. Palisade grouping is common in smears from cultures. There has always been considerable confusion in distinguishing between B. acidophilus and B. bifidus morphologically because of the fact that the latter bacillus does not always show the bifid form in cultures, whereas the former at times shows branching forms. Howe [11] also claims that B. bifidus is not necessarily an anaerobe as is claimed by Kendall and others. Noguchi,[12] in his study of the pleomorphism and pleobiosis of B. bifidus, came to the conclusion that this organism is an anaerobic phase of life of an aerobic sporogenous bacterium belonging to the subtiloid group and closely resembling B. mesentericus. By cultural procedures he induced sporulation in the aerobic phase and then was able to lead the culture back to the anaerobic bifurcating type. If his view is correct, continued cultivation under aerobic conditions should bring the sporulating stages into prominence and thus prove the identity of the culture.

All of my strains have been grown aerobically in whey agar for a period of from 4 to 18 months. I have never found bifid forms, and none of them liquefies gelatin, as Noguchi claims is the case with the aerobic and semianaerobic phases of B. bifidus. Consequently, it appears reasonable to assume that none of them is B. bifidus.

[11] Jour. Med. Res., 1917, 36, p. 481.
[12] Jour. Exper. Med., 1910, 12, p. 182.

Although in most descriptions of B. acidophilus its pleomorphism is emphasized, Howe, in his work on dental caries, came to the conclusion that this characteristic of the organism had been greatly exaggerated. My observations on this point, using 76 strains of B. acidophilus and B. acidophil-aerogenes, agree with those of Howe. Gram stains from surface growths on plain infusion agar have been compared with those from stab growths of the same cultures in whey agar made 3 months later. No distinct morphologic differences could be made out in any case. Individual strains varied considerably from one another, there being short plump forms, long thick rods, short and long chains, and frequently swollen rods of various shapes. Metachromatism was pronounced in some cultures, and involution forms were common in older cultures. It is my impression that distinctions within the Moro-Tissier group, based on morphologic characteristics, are of doubtful value.

CULTURAL CHARACTERISTERICS

B. acidophilus is frequently referred to as an anaerobe, and doubtless it does develop better under some reduction of oxygen tension. In milk at least, coagulation typically begins at the bottom of the tube and progresses upward, thus giving evidence of anaerobic tendencies. However, B. acidophilus can be readily cultivated under aerobic conditions, and in my work strains have been carried aerobically for two years. B. acidophilus will grow fairly well on slants of unneutralized infusion agar, it being a rare thing in my experience to find a strain that will not do so.

Most strains of aciduric bacteria grow poorly on gelatin, and B. acidophilus, B. bulgaricus and B. acidophil-aerogenes are unable to cause liquefaction. Noguchi claims that B. bfidus in its aerobic and semi-anaerobic phases liquefies gelatin, but Tissier states that in its anaerobic phase at least, it does not. My strains were tested in autolyzed yeast gelatin having a reaction of P_H 7. Stab inoculations were made with incubation at 37 C. Eighty strains were tested in this manner, and in no case was there more than a sparse growth, and there was no evidence of liquefaction. B. subtilis, however, grew well on the yeast gelatin and liquefied it readily.

The most common effect of B. acidophilus in milk is the formation of a smooth, compact coagulum with the appearance of little or no whey. There is a decolorization first at the bottom of the tube, gradually extending to the upper part. The time of clotting varies; some acidurics usually clot the milk within 24 hours (B. bulgaricus); others

(B. acidophilus of Moro) within from 48 hours to several days, while some strains (B. acidophilus of Finkelstein) do not clot milk. B. acidophil-aerogenes grows poorly in milk, some strains having no effect while others cause only partial clotting in from 15 to 20 days. Torrey and Rahe found that repeated transfers in milk did not shorten the clotting time in such cases. In the present study, 62 strains of B. acidophilus and 35 strains of B. acidophil-aerogenes have been tested in litmus milk at 37 C. The results are shown in Table 1.

TABLE 1

REACTION IN LITMUS MILK

	B. acidophilus	B. acidophil-aerogenes
No. strains tested	62	35
No. clotting milk in 12 days	54	6
No. clotting milk in 35 days	56	13
No. not clotting milk in 35 days	6	22

The initial hydrogen-ion concentrations favorable for the development of these aciduric bacteria have been determined as follows: 2% dextrose yeast broth was divided into 6 lots covering a range of P_H 4.2 to P_H 8.2. These were tubed in equal amounts and sterilized. Then, using a 3 mm. loop, one loopful of a broth culture was inoculated into each of the 6 broths; these were incubated at 37 C. and the turbidity of growth determined at intervals of a few hours. Seventeen strains of B. acidophilus and 3 of B. acidophil-aerogenes have been tested in this way. Table 2 shows an average result.

TABLE 2

EFFECT OF REACTION ON GROWTH
B. ACIDOPHILUS (15a)

P_H	Growth after				
	5 Hours	11 Hours	22 Hours	26 Hours	48 Hours
6.2	—	—	++	++	+++
4.8 - 5.0	—	—	+	++	+++
6.8 - 7.0	—	—	+	++	+++
4.2 - 4.4	—	—	—	—	—
7.4 - 7.6	—	—	—	++	++
8.2	—	—	—	—	—

Individual strains varied somewhat, some growing best within a range of P_H 6.2 to 7.6, but most of the cultures grew well in a range from P_H 4.8 to P_H 7.6. No strain grew at a P_H of 8.2, but 4 developed in an initial reaction of P_H 4.2. The optimum range in general appeared to be from P_H 6 to P_H 7.

BACTERIAL ANTIBIOSIS

Studies on the factors controlling the intestinal flora have shown that the predominant groups of intestinal bacteria are largely determined by the chemical nature of the food consumed. Sufficient quantities of lactose or dextrin in the diet will lead to a diminution in the number of B. coli and an enormous increase in that of B. acidophilus. Rettger [13] believes this to be the result of the slow absorption of lactose and dextrin, which thus reach the lower intestinal levels and furnish "optimum cultural and environmental conditions" for the development of B. acidophilus. Kendall [14] on the other hand, believes that the carbohydrates reach the lower levels and are there acted on by the bacteria of both groups, tending to create an acid medium which is unfavorable for the proteolytic types, and hence leads to their diminution or elimi-

TABLE 3

BEHAVIOR OF B. ACIDOPHILUS AND B. COLI, GROWN TOGETHER

Flask (lot)	1	2	3	4	5
P_H—initial C-A ratio	7.6 99-1	7.0 99-1	6.0 99-1	5.0 99-1	4.4 99-1
P_H—10 hours C-A ratio	5.8 1,000s:1	4.8 1,000s:1	4 4 50:50	4.6 40:60	4.4 No B. coli colonies Few B. acidophilus
P_H—24 hours C-A ratio	6.4 1,000s:1	4.4 1:1,000s	4.4 1:1,000s	4.4 1:1,000s	4.4 Sterile
P_H—72 hours	8.0				

nation. I [15] have shown that the simplification of the intestinal flora in white rats varies directly with the hydrogen-ion concentration of the intestinal contents and that an actual acidity of less than a P_H 5 may be present in the cecum of animals fed meat and lactose. B. coli does not develop in vitro at a lower range than P_H 5, so that apparently such conditions should be unfavorable for the growth of the usual colon groups.

The following experiment was performed to test out the behavior of B. acidophilus and B. coli when grown together in vitro. One per cent. dextrose yeast broth was used. So far as "optimum cultural and environmental conditions" are concerned, this apparently offered more favorable conditions for B. coli than for B. acidophilus in a range of P_H 6.0 to 7.6, as the former developed well within 4 hours, whereas

[13] Rettger and Cheplin: Transformation of the Intestinal Flora, 1921.

[14] Text-Book of Bacteriology, 1921, p. 625.

[15] Cannon, Paul R., and McNease, B. W.: Jour. Infect. Dis., 1923, 32, p. 175.

visible growth with the latter was not seen for 10 hours. The dextrose yeast broth was divided into 5 lots as follows: lot 1, P_H 7.6; lot 2, P_H 7.0; lot 3, P_H 6.0; lot 4, P_H 5.0; lot 5, P_H 4.4. After sterilization each lot was inoculated with a loopful of a broth culture of B. acidophilus and also with one of B. coli, thoroughly mixed, and a loopful of the mixture plated into whey agar to determine the colon-acidophilus ratio. Then the flasks were incubated at 37 C. and at intervals were retested, both as to P_H and C.-A ratio. The results are shown in table 3.

As shown in table 3, when the actual acidity developed to less than P_H 5, B. coli was practically eliminated, whereas B. acidophilus was present in great numbers. In flask 1, the dextrose was apparently used up before an acidity of P_H 5 was reached, so that at the end of 24 hours the P_H was 6.4, and B. coli was present in great numbers with B. acidophilus practically eliminated. The contents of this flask then reverted to alkalinity at the end of 3 days.

PERMANENCE OF FERMENTATIVE CHARACTERISTICS

Rahe,[8] in his report on the classification of aciduric bacteria, presented results secured from the use of freshly isolated strains. It is obviously of importance to determine whether or not such characteristics persist after prolonged cultivation. Rogers and Davis,[16] in their investigation of lactic acid bacteria, came to the conclusion that variations in fermentative characteristics are uncommon. In no case, with 150 cultures, did they find any change in fermentative activity. They carried one culture which was unable to ferment saccharose through 100 generations on saccharose agar, but at the end of this time the culture was still unable to ferment saccharose.

I have studied this question with 18 strains of B. acidophilus and 4 of B. acidophil-aerogenes by comparing their original fermentative abilities when first isolated with those at the end of from 9 to 12 months. Between the 1st and 2d tests the organisms were grown in whey agar, being transferred about every 2 weeks. Fermentation reactions were checked in 17 different carbohydrates; in 326 tests, only 3 strains showed any variation from their original reactions. One (4b) originally fermented maltose but has not done so recently in 5 attempts; a second (12b) fermented levulose and xylose when originally isolated, but does not do so now; and the third (13a) at first fermented raffinose, but now has apparently lost that power. It is evident, then, that the

[16] U. S. Dept. Agric., Bureau of Animal Industry, Bull. 154.

original fermentative powers of these strains are not lost to any appreciable extent by prolonged cultivation (one year) and that the classification of types, using freshly isolated cultures, is reasonably accurate.

PERMANENCE OF ACIDURIC CHARACTERISTICS

Kendall classifies the aciduric bacteria into two groups: the obligate and the facultatively aciduric. The latter lose their acid-resisting ability after cultivation in ordinary mediums, whereas the former do not. The permanence of aciduric tendencies was tested with 65 strains of B. acidophilus and B. acidophil-aerogenes which had been grown on whey agar for from 4 to 12 months, 22 strains having been isolated at least 10 months previously. Growth occurred in every case when they

TABLE 4

B. ACIDOPHILUS

Type	Maltose	Glucose	Lactose	Sucrose	Raffinose	Milk Clotting
A	+	+	+	+	+	++
B	+	+	+	+	—	++
C	+	+	—	+	—	— ?
D	+	+	—	—	—	++

B. ACIDOPHIL-AEROGENES

A	g	g	g	g	g	++
B	g	g	g	g	+	++
C	g	g	g	g	—	++
D	g	g	+	g	+	++
E	g	g	—	g	—	— ?
F	g	g	—	—	—	+ ?
G	g	g	+	+ g	g	++
H	g	g	g	+	g	++

were inoculated into dextrose yeast broth with an initial reaction of P_H 4.8, thus showing the persistence of their original aciduric characteristics.

CARBOHYDRATE FERMENTATION

The members of this group are carbohydrophilic and will develop poorly, if at all, in the absence of a utilizable carbohydrate. Attempts to classify various types have thus depended largely on fermentation tests. The most recent research along this line was made by Rahe.[8] He divided B. acidophilus into 4 subtypes and B. acidophil-aerogenes into 6, based on their abilities to ferment certain carbohydrates. Rahe's scheme of classification is shown in table 4.

I have tested the following carbohydrates: dextrose, lactose, saccharose, maltose, galactose, levulose, mannitol, mannose, rhamnose,

dulcitol, trehalose, arabinose, xylose, dextrin, inulin, raffinose, melezitose, glycerol, salicin and starch. In interpreting the results obtained, it should be emphasized that no organism has been considered a fermenter unless it has produced a reaction of p_H 5 in autolyzed yeast carbohydrate broth. Furthermore, no organism has been considered unable to ferment a carbohydrate until 3 negative results have been obtained.

Summarized results of the fermentation tests are as follows:

A. Monosaccharides.
  1. Hexoses.
    (a) Dextrose, levulose, galactose.
      Fermented by all strains of B. acidophilus (60) and all of B. B. acidophil-aerogenes (35).
    (b) Mannose.
      Fermented by 58 of 64 strains of B. acidophilus and 10 of 29 strains of B. acidophil-aerogenes.
  2. Pentoses.
    (a) Arabinose.
      Fermented by 17 of 65 strains of B. acidophilus and 19 of 35 strains of B. acidophil-aerogenes.
    (b) Xylose.
      Fermented by 8 of 56 strains of B. acidophilus and 16 of 33 strains of B. acidophil-aerogenes.
    (c) Rhamnose.
      Fermented by 20 of 63 strains of B. acidophilus and 1 of 35 strains of B. acidophil-aerogenes.

B. Disaccharides.
  1. Maltose.
    Fermented by all strains of B. acidophilus (63) and by all strains of B. acidophil-aerogenes (35). If B. bulgaricus is unable to ferment maltose, as claimed by Rahe and others, these results add weight to Rahe's claim that B. bulgaricus is not an inhabitant of the digestive tract.
  2. Lactose.
    Fermented by 62 of 65 strains of B. acidophilus and by all of the strains of B. acidophil-aerogenes (35).
  3. Sucrose.
    Fermented by 62 of 64 strains of B. acidophilus and 34 of 35 B. acidophil-aerogenes.
  4. Trehalose.
    Fermented by 53 of 65 strains of B. acidophilus and by 3 of 35 of B. acidophil-aerogenes.

C. Trisaccharides.
  1. Raffinose.
    Fermented by 36 of 66 strains of B. acidophilus and by all of B. acidophil-aerogenes (35).
  2. Melezitose.
    Fermented by 30 of 64 strains of B. acidophilus and by 1 of 35 of B. acidophil-aerogenes.

D. Polysaccharides.

1. Dextrin.

Fermented by 8 of 65 strains of B. acidophilus and 1 of 35 of B. acidophil-aerogenes. These results would suggest that the beneficial effects of this carbohydrate in transforming the intestinal flora must lie in the slow absorption and probably partial hydrolysis before reaching the lower digestive levels.

2. Starch (Potato).

Fermented by 3 of 54 strains of B. acidophilus and by none of 26 strains of B. acidophil-aerogenes. These 3 strains also fermented dextrin, but not inulin.

3. Inulin.

Fermented by 4 of 60 strains of B. acidophilus and by 1 of 30 of B. acidophil-aerogenes.

TABLE 5

CLASSIFICATION ACCORDING TO SOURCE
B. ACIDOPHILUS

Number of Strains	Origin	Rahe's Types			
		A	B	C	D
37	Intestinal tract.....	24	10	2	1
23	Sputum.....	8	15	0	0
4	Sour milk.....	2	2	0	0

B. ACIDOPHIL-AEROGENES

		A	B	C	D	E	F	G	H
18	Intestinal tract.....	15	1	0	1	0	0	0	1
16	Sputum.....	14	0	0	1	0	0	0	1

E. Alcohols.

1. Mannitol.

Fermented by 44 of 64 strains of B. acidophilus and by 2 of 35 of B. acidophil-aerogenes. This differs from the observations of Rahe, who found mannitol a poor test substance. Perhaps my results are attributable to the apparent superiority of autolyzed yeast broth to unneutralized sugar-free broth as a basis for the tests.

2. Dulcitol.

Fermented by 11 of 65 strains of B. acidophilus and by 1 of 35 of B. acidophil-aerogenes. Each of fermenting strains of B. acidophilus also fermented mannitol and the fermenting culture of B. acidophil-aerogenes also fermented mannitol and inulin.

3. Glycerol.

Fermented by 6 of 54 strains of B. acidophilus and by none of 26 strains of B. acidophil-aerogenes. The 6 strains of B. acidophilus which fermented glycerol also fermented mannitol.

F. Glucosides.

1. Salicin.

Fermented by 42 of 54 strains of B. acidophilus and by 1 of 26 of B. acidophil-aerogenes. The fermenting strain of B. acidophil-aerogenes also fermented inulin, mannitol and dulcitol.

CLASSIFICATION

The strains used in the present study are classified in table 5 in accordance with the scheme of Rahe.

SUMMARY

A biologic study of 64 strains of B. acidophilus and 34 of B. acidophil-aerogenes was made. These organisms were isolated from 93 different sources, mostly from the sputum and feces of normal human adults. Dried autolyzed yeast was used as the basis for all the fermentation tests, and was particularly satisfactory for the study of these members of the aciduric group.

These bacteria are facultative anaerobes, but grow well under aerobic conditions. They do not liquefy gelatin, and may or may not clot milk. They grow best in a reaction of P_H 6 to 7, but will develop within the range of P_H 5.0 to 7.6 at least. When grown in dextrose yeast broth in mixed culture with B. coli, with an initial reaction of P_H 7.0 to 7.6, B. coli at first gains the ascendency, but B. acidophilus becomes predominant as the P_H approaches 5.

In the case of 22 strains, both aciduric and fermentative characteristics were practically constant for 12 months. The fermentation reactions for 20 carbohydrates were determined, all negative tests being checked at least twice.

A classification according to the scheme of Rahe is given.

STUDIES ON RESPIRATORY DISEASES

XVI. FURTHER OBSERVATIONS ON THE CHARACTERISTICS AND OCCURRENCE OF THE HEMOPHILIC BACILLI *

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The so-called hemophilic bacteria are now generally recognized as constituting a large group of organisms, apparently all parasitic on the mucous membranes of warm-blooded animals. Up to the time of the influenza epidemic of 1918, little was known about their character and distribution, but the recent large volume of intensive work on the Pfeiffer bacillus, long regarded by many as the causal agent of influenza, has added much to our knowledge of the whole group. Since several more or less complete summaries of the literature have already appeared, this paper is devoted to observations that supplement or extend our existing information.

MORPHOLOGY

In the study of morphology, 72 strains have been under observation, 29 from clinical influenza cases, 15 from throats of persons with common colds, 13 from cases of tonsillitis and 15 from normal throats; 8 of these strains were isolated during the pandemic of 1918-1919, and the remaining 64 since September, 1919. All these strains were carefully tested for hemophilic qualities, colony appearance, negative gramstain, and satellite growth with a standard strain of staphylococcus. Hemolytic organisms were not included in this particular series and will be dealt with in another part of the paper.

Microscopic examinations were made of smears from 24-hour and 7-day growths on 5 different culture mediums (table 1), and each examination was duplicated from cultures grown at other times. At least 3 different fields were examined in each smear. Observations were recorded each time without reference to previous records. Each strain was examined microscopically in this way at least 20 times.

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

Five morphologic types could be distinguished (fig. 1). The most common form (fig. 1, *A*) is that which has been regarded by most observers as characteristic of the Pfeiffer bacillus, a very small, short, thick rod with rounded ends; it frequently occurs in pairs with the distal ends tapering; in liquid mediums short chains of 3 to 6 bacilli have been found. Cells of this type are probably present in all strains, although they may not be found in all smears. They were found in considerable numbers in strains predominantly of the filamentous type, but might readily have been overlooked, since the odd shapes of the larger organisms tend to engross the attention of the observer. Of the 72 strains under observation, type A alone was found in 33 and was the predominant type in 18 others.

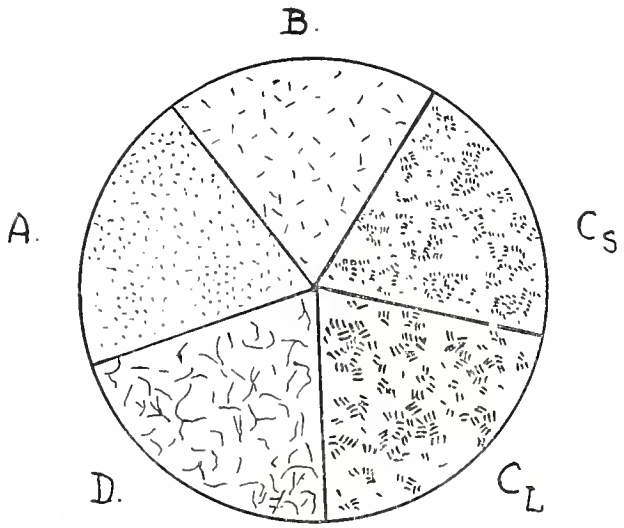

Fig. 1.—Diagram of morphological types of Pfeiffer bacilli.

Type B is apparently the one to which Pfeiffer at first gave the name of pseudo-influenza bacillus. It is longer and thicker than type A, often curved, and less uniform in size and shape. It has not been found in pairs or chains. It was observed in 17 of the 72 strains, but never as the sole type on all mediums of all ages. Since this type is relatively infrequent and is generally found in the presence of the true filamentous type, it may perhaps be a stage in the change from a short rod to a long and branched one.

Type C is here used to designate microscopic pictures with a definite cell arrangement in clusters or groups. Pfeiffer's original description reads "die Stäbchen liegen meist nester- und häufenweise." While we

have observed definite grouping relatively seldom, parallel aggregations of the bacilli sometimes occur with a constancy and definiteness worthy of remark. Two sizes of cells were observed, the smaller (C_S) practically identical individually with type A and the larger, somewhat thicker and shorter than type B (C_L). There seems little doubt that types A and C_S are closely related, and the sole justification for considering the latter as in any way independent is its frequent occurrence in the smears of certain strains and its uniform absence in others. To obtain uniformity in our records the rule was followed that all small-cell smears showing parallelism, distinct or slight, were recorded as C_S, and if many single ungrouped rods were found in the same smear, both types were recorded as present (A C_S). Tabulated in this way it soon became apparent that the parallel aggregations or nests occurred frequently in some strains of the A type, in others never (table 1).

The larger cells (C_L) in similar grouping were still less commonly found. They evidently bear the same relation to the B type that C_S does to the A type.

Type D includes the irregular filamentous forms called pseudo-influenza bacilli by some writers. Curved and occasionally branching forms occur. There is great variation in size and shape of the individual elements. The small type A cell was found associated with type D in all but 8 of the 24-hour smears. Type D was present at some time on some medium in 26 of the 72 strains.

Table 1 illustrates the kind of result obtained on various mediums and at different times. In each case the two 24-hour and 7-day records were independent observations made at considerable intervals and without reference to findings on previous smears. The results on all strains may be summarized as follows: More of the irregular forms (type D) were found in 7-day growths than in 24-hour growths.

If we include all smears in which D forms were found, either pure or mixed with other types, the numbers are 69 D types in 24-hour cultures, 94 in 7-day cultures.

The nature of the medium does not seem to affect the morphology greatly, if type A is assumed to be the normal form (table 3).

Hydrogen-ion concentration was at first thought to be of great importance, and throughout all the series illustrated in table 1 was carefully adjusted. Later experiments with chocolate-agar medium of different P_H concentrations, ranging from P_H 6.4- 8.8 showed that while the luxuriance of growth was affected at the extremes, morphology

TABLE 1

Occurrence of Morphologic Types of the Pfeiffer Bacillus (72 Strains) on Different Mediums (Ph 7.6)

Strain	Chocolate-Blood Agar				Blood Agar				Oleate Medium				Blood Broth				Blood Milk			
	24-Hour		7-Day		24-Hour		7-Day		24-Hour		7-Day		24-Hour		7-Day		24-Hour		7-Day	
06 and 32 other strains	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
0 2 1	C_S	C_S	A	A	C_S	C_S	A	C_S	C_S	C_S	AB	C_S	AB	A	A	A	AC_S		A	AC_S
0 2 2	BD	ABC_SD	BD	ABD	ABC_SD	ABD	ABC_SD	ABC_SD	A	AC_LD	AD	BD	ABD	AB	AD	D	BD	AD	AD	D
G L I T Ba	C_S	C_L	C_S	AC_L	C_S	C_SD	C_SC_S	D	AC_S	D	AC_S	D	AC_S	D	AC_S	D	AC_S	AC_S	C_S	AC_S

was little, if at all, affected. Strain GLITOb, for example, showed pure type A morphology on chocolate-agar medium of the following P_H concentrations: 6.4, 6.7, 7.0, 7.4, 7.8, 8.1, 8.4, 8.6 and 8.8. Sixteen strains grown on medium of three different P_H values were not markedly affected, although there was a slight but undoubted tendency to the production of type D forms at the two extremes. If the occurrence of type A be considered normal and that of type D abnormal, a hydrogen-ion concentration of P_H 7.4 would rank as the most favorable.

The frequency of the several types appears connected in some degree with the age of the strain. Freshly isolated strains are more apt to show pure type A, while type D appears more commonly in strains long under cultivation. Some strains, however, have shown pure type A on all culture mediums for over a year after isolation.

TABLE 2

RESULTS IN 24-HOUR AND 7-DAY CULTURES

Type	24-Hour Cultures	7-Day Cultures
A only	512	505
B only	3	2
C_S only	33	24
C_L only	16	9
D only	8	23

TABLE 3

PURE TYPE A (WITHOUT ADMIXTURE) FOUND

Medium	Number of Smears
Chocolate agar	213
Blood agar	203
Oleate medium	177
Blood broth	208
Blood milk	216

Cultures obtained from the heart blood of mice, each inoculated with a "chocolate-agar slant" of a different strain gave results immediately after isolation as follows: ABC_LD, AC_S, ABD, A, AC_S, A, A, C_L, findings very similar to those obtained with strains never subjected to animal passage.

No significant difference in morphology was observed in strains of different origin. Strains from normal throats, from human organs at necropsy and from the nasopharynx of patients with epidemic influenza were morphologically indistinguishable.

The true meaning of the somewhat extreme morphologic variations among strains of the Pfeiffer bacillus is not clear. The various forms

observed are unquestionably not due primarily to differences in the nutrients or in the hydrogen-ion concentraiton of culture mediums or in the age of the culture, although all these factors have some influence. It is as difficult to explain the marked persistence of a pure morphologic type in some cases as the mixture and apparent variability in others (table 1). The extent to which the several types breed true can of course be determined only by single cell isolation, and this we have not attempted. Our observations do not lend support to the assumption that the more unusual types in these bacterial cultures betoken the development of "sex cells." The dominance and persistence of the several morphologic types appear to be strain characteristics rather than due to environmental causes or to "cycles of development."

INDOL PRODUCTION

In the course of this work 220 strains [1] have been tested for indol production; 126 (57%) of these have been positive, 94 (43%) negative. The methods used have been the same as those described in an earlier paper.[2] Our observations show that original indol-producing strains continue to produce indol in successive subcultures; in the case of some strains this quality has persisted over a 6-year period. Conversely, strains that have not produced indol when first isolated have not acquired this power. Strains recovered from inoculated mice continue to manifest their original characters. Indol production is at least as permanent and significant a property as other biologic qualities commonly used for differential tests. Similar observations, although not extending over so long a time, have been made by several workers.[3]

As appears in the following table (table 4), indol + and indol — strains were found with about equal frequency in the throats of apparently normal persons.[4] In persons suffering from sore throat or colds at the time of examination, however, indol-positive strains were found more frequently than indol-negative strains. It may also be noted that most of the strains obtained from influenza sputum in 1918-1919 and

[1] Duplicate cultures from the same individual have been eliminated in all our tahulations, but there are a few (less than a dozen) instances in which strains with different characters have been obtained from the same plate, and these strains are included in our series.

[2] Jordan: Jour. Am. Med. Assn., 1919, 72, p. 1542.

[3] Suzuki, N.: Studies of Influenza, Gov't Inst. for Infect. Dis., Tokio, Dec. 28, 1922, p. 51; Kunizaki, T.: Ihid., p. 53; Kristensen, M.: Occurrence and Classification of Hemoglobinophilic Bacteria, Copenhagen, 1922.

[4] The cultures secured from persons with normal throats and with colds were mostly ohtained from throat swabbings of University of Chicago students. These observations on normal persons and on persons with colds cover the same time period and are quite comparable with respect to age grouping, contact and other factors, since they were made mostly on students in our laboratory classes and were taken in routine series.

in the spring of 1920 were indol +, as were likewise all 3 strains obtained from human necropsies, one from the lung, one from the heart blood after fatal influenza and a third from meningitis. Rivers [5] has pointed out that the great majority of meningitis strains are indol producers.

OTHER CULTURAL CHARACTERS

Nitrate reduction in these organisms seems to have been first observed by one of us (E. O. J.),[6] and was later confirmed by Stillman and Bourn.[7] Our further observations have established the importance of this character as a differential criterion. Of 287 strains of the Pfeiffer bacillus thus far tested, all have reduced nitrite to nitrate, while B. pertussis (6 strains) and B. bronchisepticus (3 strains) have lacked this power.

TABLE 4

SOURCE OF INDOL + AND INDOL — STRAINS

	Indol +	Indol —
Influenza	14	4
Necropsies	3	0
Pneumonia	6	4
Colds	43	28
Normal	59	57
Unknown	1	1
	126	94

Fermentation tests with hemoglobinophilic bacilli have not proved satisfactory for differential purposes. Most observers have obtained irregular and not very well defined results.[8] Kristensen [9] was unable to demonstrate any certain power of fermentation, either in solid or liquid mediums. Stillman and Bourn,[7] however, found that acid was generally produced in suitable dextrose mediums, and their results have been confirmed by others. Our own observations are quite in accord with these findings. Only 9 out of 113 strains tested in peptone-salt-horse-blood medium (P_H 7.4) with 1% of dextrose have failed to give evidence of fermentation (Andrade indicator). Some strains, indeed, were able to produce gas quite consistently in dextrose agar.

We have found a favorable medium to be veal infusion broth (P_H 7.4), 1% agar, 1% dextrose, 1% Andrade indicator, autoclaved,

[5] Jour. Am. Med. Assn., 1920, 75, p. 1495; Am. Jour. Dis. Child., 1922, 24, p. 102; Jour. Exper. Med., 1921, 34, p. 477.

[6] Jordan: Am. Jour. Pub. Health, 1920, 10, p. 648.

[7] J. Exper. Med., 1920, 32, p. 665.

[8] Povitzky, O. R., and Denny, H. T.: Jour. Immunol., 1921, 6, p. 65; Rivers, T. M., and Kohn, L. A.: Jour. Exper. Med., 1921, 34, p. 477.

[9] Ocurrence and Classification of Hemoglobinophilic Bacteria, p. 163.

2% horse blood added, tubed aseptically, heated in water bath at 60 C. for 3 hours, cooled and inoculated with a deep stab. Of 165 strains tested in this medium, 36 (22%) have produced gas. This is a somewhat higher proportion of gas formers than that observed by Stillman and Bourn (13%). There is undoubtedly irregularity in the behavior of these organisms. Duplicate tubes were inoculated in each test, and in several instances acid or gas formation was observed in one tube while its fellow showed no change. It is manifest that this is a crude method of measuring gas production and that the use of the Eldredge tube recommended by Ayers, Rupp and Mudge[10] for determining carbon dioxide production might be expected to give more accurate results. Six strains that had never given evidence of gas production by the ordinary method were tested by the Eldredge tube method, and all showed carbon dioxide to be produced in the Ayers, Rupp and Mudge medium (with the addition of 2 horse blood). In all tubes the purity of the culture was rigorously controlled by preliminary incubation and by culture tests made after 5 days' growth.

TABLE 5

CHARACTERS OF NONHEMOLYTIC PFEIFFER BACILLUS STRAINS; PERCENTAGE GROUPING

	A	B	C	D	E	F	G	H
Indol	+	—	—	—	+	—	+	+
Saccharose fermentation	—	+	+	—	—	—	+	+
Gas in dextrose medium	—	+	—	—	+	+	—	+
Stillman and Bourn percentage (119 strains)	52	12	11	21	0	1	1	0
Jordan and Reith percentage (165 strains)	35	8	6	29	6	6	8	2

or	Indol +	Saccharose +	Gas in dextrose agar
Stillman and Bourn	53	24	13
Jordan and Reith	51	24	22

Saccharose fermentation is likewise variable and uncertain, and a larger proportion of the tests results negatively than with dextrose. Two fluid mediums (7.4) have been carefully tried out, both containing 1% saccharose and 2% Armour's peptone and 0.5% NaCl. The latter has given a considerably larger number of positive results. In all, 24% of 165 strains have fermented saccharose. This is exactly the same percentage of saccharose fermentation as that noted by Stillman and Bourn.

Table 5 shows the relative grouping of characters in our 165 strains compared with the observations of Stillman and Bourn (119 strains).

We have not been able to correlate fermentation characters with origin, age, or agglutinative affinities[11] of the various strains. On

[10] Jour. Infect. Dis., 1921, 29, p. 235.

[11] Jordan and Sharp: Ibid., 1922, 31, p. 198.

several occasions representatives of two or more groups have been obtained from the same throat swab.

Milk-blood-broth is an excellent medium for the growth of the Pfeiffer bacillus, but with either brom cresol purple or litmus as indicator, the reactions are irregular. The great majority of strains give a slightly alkaline reaction, but occasional strains give consistently a slightly acid reaction on the same lot of medium in which the majority produce alkali. Strains of B. pertussis and B. bronchisepticus can be readily picked out of any series by their much more energetic alkali production. Our results with milk cultures are essentially similar to those already reported by one of the writers,[6] and by Rivers and Kohn.[12] The medium is not very useful for differentiation.

OCCURRENCE OF THE PFEIFFER BACILLUS IN NORMAL THROATS

Among the several lines of evidence thought to bear on the significance of the Pfeiffer bacillus in epidemic influenza; the relative incidence in healthy persons at different times and places has been the subject of considerable investigation. It is plain, however, that the reported numerical results are not of equal weight. Different degrees of skill and experience on the part of the observers and especially differences in culture mediums have unquestionably influenced the findings to such an extent that strict comparability is impossible. An important steadying factor came into play with the introduction of Avery's oleate hemoglobin agar.[13]

In the course of a 12-month serial study here reported[14] comparative plates were made from the nasopharyngeal swabs on both oleate medium and horse-blood agar. The blood agar was streaked with staphylococcus, since our observation has shown that the favoring effect of the "satellite" action gives a larger percentage of isolations. The oleate medium was made with a hydrogen-ion concentration of P_H 7.2-7.5, which experience has shown to be most favorable.[15] Every fresh lot of medium (both oleate and blood agar) was tested with recently isolated strains of Pfeiffer bacilli, and controls were made at each plating. These precautions are essential in comparative work. Selection of Pfeiffer-like colonies from the plates was always made by an adept of long experience. The data on record cards were not tabulated until the completion of the whole series. In the first 120 cases examined—

[12] Jour. Exper. Med., 1921, 34, p. 477.
[13] Jour. Am. Med. Assn., 1918, 71, p. 2050.
[14] We have been assisted in this work by Miss Ruth Westlund.
[15] Cf. also Winchell and Stillman: Jour. Exper. Med., 1919, 30, p. 497.

persons with normal throats and with colds—plates were made on both mediums. The Pfeiffer bacillus was isolated in 31; from both blood and oleate mediums in 15 cases; from blood agar alone in 3; from oleate medium alone in 13. The number of colonies picked from blood agar was 92, 38 of which, or 41%, proved to be Pfeiffer bacilli, while out of 119 colonies picked from oleate medium, 68 or 57% were positively identified. These figures demonstrate the superiority of the oleate medium as a means for isolating the Pfeiffer bacillus, even when the blood agar medium is used under favorable conditions by those who have had prolonged experience with it.

Although as late as 1919 students of influenza felt able to say that "there is inadequate proof that the Pfeiffer bacillus is a member of the normal upper respiratory tract flora," [16] subsequent observation indicated that typical Pfeiffer bacilli occur in perfectly normal persons, both as transient and as more or less permanent elements of the respiratory flora.[17] Wollstein and Spence,[18] however, express the opinion that an exceedingly small number of healthy persons carry the Pfeiffer bacilli in their respiratory tract "even during the time of an influenza epidemic," unless there is a history of direct exposure. Bloomfield,[19] as the result of a comprehensive series of studies, concludes that the Pfeiffer bacilli have no more than a moderate and partial degree of adaptation to free growth on normal mucous membranes. There seemed thus reason for further examination of the normal throat flora to determine the presence, abundance and seasonal prevalence of the Pfeiffer bacillus.

During the calendar year 1922 plates were made from the throats of 294 University students (about one-fifth being women) according to methods previously described,[20] oleate medium being used throughout. With the first 120 examined, blood agar was also used as already stated. At the time of examination 271 of these persons were in apparently normal health, and the throat appeared to be in healthy condition. The Pfeiffer bacillus [21] was found in 108, or 40%, of the throats of these seemingly normal persons. In most instances in which positive findings were obtained, the number of Pfeiffer-like colonies on the plates was

[16] Duval and Harris: Jour. Infect. Dis., 1919, 25, p. 384.

[17] Pritchett and Stillman: Jour. Exper. Med., 1919, 29, p. 259; Winchell and Stillman: Ibid., 1919, 30, p. 497; Pilot and Pearlman: Jour. Infect. Dis., 1921, 29, p. 55.

[18] Am. Jour. Dis. Child., 1920, 19, p. 459.

[19] Bull. Johns Hopkins Hosp., 1922, 33, p. 172.

[20] Jordan, E. O.: Public Health Rept., 1919, 34, p. 1413.

[21] Both hemolytic and nonhemolytic types are included in the following summary. A separate analysis of the significance of the hemolytic type will be made later in this paper.

recorded as "several," "few," or "very few," but in about 10% there were "many" Pfeiffer-like colonies.

Information was obtained from 290 of the 294 examined regarding influenzal history. The data were probably as accurate as could be obtained from any group. The results are given in table 6.

TABLE 6

INFLUENZAL HISTORY OF PERSONS EXAMINED FOR PFEIFFER BACILLI

	Number	Pfeiffer Bacilli Found In	%
No history of influenza	173	72	41
Influenza in 1918-19 epidemic	76	38	50
No influenza in 1918-19, but clinical influenza later*	41	23	56

* Sharply differentiated from an ordinary cold in the opinion of the persons reporting.

No difference appeared in these groups in the relative abundance of Pfeiffer bacillus colonies on the plates. There was as large a proportion of plates with "many" Pfeiffer bacillus colonies in the first group as in the second.

The relation between colds and the presence of Pfeiffer bacilli was shown by the figures in table 7.

TABLE 7*

RELATION BETWEEN COLDS AND PFEIFFER BACILLI

	Number	Pfeiffer Bacilli Found	Percentage
Cold in progress at time throat was swabbed	64	37	58
Recent cold (within 1 month)	47	22	47
No cold within 1 month	95	39	41
No cold within 6 months	72	26	36
Chronic rhinitis	12	4	..

* One student reported never having colds, 2 had hay-fever at the time of examination, one had been gassed and his throat was still affected; in none of these was the Pfeiffer bacillus found.

Inspection of the individual throats at the time of swabbing was confirmatory of the findings in the table.

TABLE 8

PFEIFFER BACILLI FOUND IN NORMAL AND IN INFLAMED THROATS

	Number	Pfeiffer Bacilli Found	Percentage
Throat apparently normal at time of examinatio..	271	108	40
Throat visibly inflamed at time of examination..	22	12	54

The seasonal distribution of the Pfeiffer bacillus in persons showing no sign of respiratory disease at the time of examination appears in table 9.

TABLE 9*

RESULTS OF EXAMINATION OF THE THROATS OF HEALTHY PERSONS AS TO THE PRESENCE OF PFEIFFER'S BACILLUS

1922	Positive	Negative
January	8	20
February	6	15
March	11	4
April	11	12
Mary	5	4
June	8	7
July	14	15
August	4	33
(September)	11	17 (O. F.)
October	6	14
November	11	9
December	7	3

* The data, as before, are derived from the examination of University of Chicago students, except the figures for the month of September. The figures for that month are from throat swabbings of adults at the Oak Forest Infirmary. The student body is a fairly uniform age group throughout the year, except in the summer months when it is composed largely of older persons who live outside of Chicago the rest of the year. The July and Augusta data relate to the latter group. The University is closed in September.

Two points in table 9 deserve notice: the large proportion of positive findings in March, at a time when an outbreak of respiratory disease generally regarded as influenza was widely prevalent through the country,[22] and the very low incidence of Pfeiffer's bacillus in the August throat examination.

Whether the relative scarcity of Pfeiffer bacilli in the summer months is a seasonal effect or whether it is due to the large proportion of students in July and August who had been previously smalltown dwellers is uncertain.

The great frequency of the Pfeiffer bacillus in normal throats at the period of the 1922 influenza outbreak may have some significance. It is supported in various ways. In January the throats of 11 persons with "colds" were examined; the Pfeiffer bacillus was present in 6, absent in 5; in March the Pfeiffer bacillus was found in every one of the 4 "cold" cases seen. The numbers are too small to have much value, but are quite in line with our observations on healthy persons. Bloomfield[19] has called attention to the apparently high incidence of Pfeiffer bacilli in the throats of healthy people during and just after

[22] The combined deaths from influenza and pneumonia (all forms) in Chicago were as follows:

Week Ending	Number of Deaths Reported
Jan. 28, 1922	65
Feb. 4, 1922	72
Feb. 11, 1922	80
Feb. 25, 1922	94
March 4, 1922	139
March 11, 1922	150
March 18, 1922	130
March 25, 1922	92
April 1, 1922	65

the 1918-1919 pandemic. Our own observations in 1922, which are strictly comparable among themselves during the period studied, seem to indicate that the Pfeiffer bacillus found unusually favorable conditions for residence on the human mucous membranes at a time when clinical influenza was prevailing.

The preceding records have been based on observations made from a single examination. Like all those who have given attention to the question, we have found that repeated throat swabs of the same person may yield results unlike those of the initial examination, a positive finding giving way to a negative and vice versa. The importance of serial examination has been especially emphasized by Bloomfield.[23] We have examined one series of 10 persons with the oleate medium, making frequent throat swabs and complete cultural identification over a period of about 2 months (Nov. 15, 1920-Jan. 21, 1921). In all but one of these (19 negative examinations) Pfeiffer bacilli were found on one or more occasions, although on the initial examination only 2 of the 10 were positive. The number found was usually small and, except in one instance, never exceeded 10% of all colonies on the plate; in this person, in good health at the time and with an apparently normal throat, Pfeiffer-like colonies constituted 50% of the total growths. In one case the Pfeiffer bacillus was found only once in 21 examinations on as many different days; in another it was found 9 times in 27 examinations. In some instances the findings were bunched as in A, in whom the Pfeiffer bacillus was found on Dec. 2, 7, and 8, but was absent between Nov. 11 and Dec. 1 (12 negative examinations) and between Dec. 9 and Jan. 14 (10 negative examinations); in other cases they were widely scattered, as in A_8 in whom the Pfeiffer bacillus was found on Nov. 17 and Jan. 11 but was absent on all other dates between Nov. 15 and Jan. 16 (25 negative examinations). Three other persons under observation for a shorter period, Jan. 5 to Jan. 21, 1921, gave results entirely comparable to those in the more extensive series. In 4 of the persons under observation, colds developed during the examination period, but in only one instance were Pfeiffer bacilli found while the cold was in progress, and then in small numbers.

HEMOLYTIC PFEIFFER BACILLI

A gram-negative bacillus of varying morphology but fairly constant cultural characters has been found growing in hemolytic colonies on blood-agar plates by several observers. Pritchett and Stillman,[17]

[23] Jour. Am. Med. Assn., 1921, 77, p. 187.

who were apparently the first to describe this organism under the designation "Bacillus X," refer to the colonies as readily confused with those of B. influenzae. Rivers and Leuschner [24] note that this or a similar organism was observed by members of the pneumonia commission working at Fort Sam Houston in 1918.

The significance of the hemolytic Pfeiffer bacilli, their cultural characters and their relation to the nonhemolytic variety have been studied by Rivers,[25] Stillman and Bourn,[7] and Bloomfield.[26] The present observations extend somewhat the results of these investigators.

The cultures used in this study have been obtained from a series of blood-agar cultures from nasopharyngeal swabs taken in the period Jan. 1, 1922, to Jan. 1, 1923. About three-fourths of the persons examined, mostly university students, were in apparently good health at the time of examination, and the upper respiratory tract appeared to be normal; the remaining one-fourth had just recovered from a cold or were suffering from a cold when the throat was swabbed.

It is probable that colonies of these organisms were encountered earlier in our work, but were discarded on account of their noteworthy divergence from the colonies of the typical nonhemolytic Pfeiffer bacillus.

Previous observers have described the hemolytic colony as resembling closely the beta type of streptococcus (Pritchett and Stillman; Rivers and Leuschner; Bloomfield). Apparently this is the only type of hemolysis recognized by most of those who have studied this organism. There is no doubt, however, that another type of hemolytic colony sometimes occurs. This we have called the delta type (fig. 2). The individual colonies of the delta type are much more minute, and the zone of hemolysis is narrow and usually not entirely freed from blood pigment. To the naked eye this type of hemolysis sometimes resembles that of the green producing streptococcus. The types breed true with a considerable degree of constancy. The beta and delta types shown on one plate in fig. 2 have retained their characters unchanged for 6 generations. Some strains that appeared to show variation have been found, by careful plating, to contain both colony types. It is not always easy to obtain sharp differentiation, and strains seemingly intermediate occur. In general, however, our strains fell definitely into one or the other group. Of 49 strains of hemolytic Pfeiffer

[24] Bull. Johns Hopkins Hosp., 1921, 32, p. 130.
[25] Ibid., 1920, 31, p. 50.
[26] Ibid., 1921, 32, p. 378.

bacilli carefully studied with respect to their hemolysis, 25 were of the beta type, 24 of the delta type. No correlated morphologic or cultural characters were observed. It seems possible that strains of the delta type have been encountered by other observers but not specifically distinguished. Stillman and Bourn mention that "an occasional strain is encountered . . . whose hemolytic powers are not well developed." Kristensen (p. 207) also reports finding a strain that gave consistently "slow and comparatively weak hemolysis" over a considerable period.

Morphologically some strains of the hemolytic Pfeiffer bacilli show no difference from the typical nonhemolytic, very small rod (type A), but as a rule, even in a young (18-24 hour) culture, longer rods and

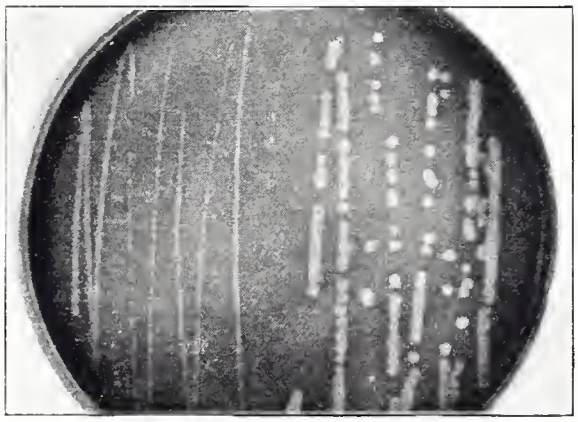

Fig. 2.—Types of hemolysin produced by hemolytic Pfeiffer bacilli.

thread forms are common. We find, like Kristensen and some other observers; that large and relatively filamentous forms are much more likely to predominate in cultures of the hemolytic than in those of the nonhemolytic type. All are gram-negative.

Colony structure of the hemolytic and nonhemolytic groups has been described by Rivers and Leuschner as "almost identical." Slight, but possibly significant, differences may, however, be observed. The hemolytic colonies are usually less translucent and are almost always distinctly gray. A young 24-hour nonhemolytic colony generally has a watery consistency, while a hemolytic colony of the same age is dry, and can usually be lifted up with the needle. The size of the colonies varies about as much as that of the nonhemolytic, although no giant colonies have been observed in hemolytic strains.

An interesting difference between the hemolytic and nonhemolytic strains is the greater fragility of the former on the usual culture mediums. Chocolate-agar, which is peculiarly favorable for most nonhemolytic strains, is poorly adapted for the hemolytic type; the latter can be kept alive more successfully by daily transfers on blood-agar. Kristensen also has noted the poor keeping quality of the hemolytic strains. This troublesome feature has made it difficult to keep under cultivation a sufficient number of strains for comparative biologic tests.

The satellite phenomenon, or "symbiosis," is usually only slightly exhibited or is absent in our hemolytic strains. Certainly it is not so striking or so characteristic a quality of this organism on blood-agar plates as it is of the nonhemolytic Pfeiffer bacilli.[27] In these observations a standard strain of staphylococcus has been used which had previously been found to give favorable and consistent results with cultures of nonhemolytic strains. The delta strains of hemolytic bacilli not infrequently showed a slight augmentation of growth in the neighborhood of the staphylococcus streak, but not nearly so marked as that shown by nonhemolytic Pfeiffer bacilli. On the same medium the beta strains never manifested satellitism. Since both types of hemolytic organisms grow fairly well all over blood-agar plates, the need of accessory growth factors, derived from staphylococcus, is evidently not imperative. When, however, we plated the beta hemolytic strains, which on blood-agar had never manifested satellitism, on peptone-salt-agar (2% Armour's peptone, 0.5% NaCl, 2% agar in tap water) and inoculated the medium at several different points with staphylococcus growth occurred in the vicinity of the staphylococcus colonies, but nowhere else on the plate. Occasionally a strain would not show the satellite phenomenon in a single plating, but all beta strains tested (about 20) have exhibited it at one time or another.

The biochemical characters of the hemolytic strains show certain deviations from the prevailing nonhemolytic types. All strains reduce nitrates to nitrites, but indol is rarely produced. Only 4 out of our 49 strains have been found to form indol. Stillman and Bourn observed only 3 indol formers among 29 hemolytic strains, and Rivers and Leuschner 3 among 13. Kristensen, on the contrary, found 14 indol-positive strains among 18 examined. A real difficulty in such tests is the frequent mixture of hemolytic and nonhemolytic types of Pfeiffer bacilli. A number of our cultures which produced hemolytic

[27] D. J. Davis has especially emphasized the differential value of the satellite phenomenon in the nonhemolytic type, Jour. Infect. Dis., 1921, 29, p. 187.

colonies and also gave indol production were plated out and found to be mixtures. One culture, for example, was plated and 20 colonies were picked, 15 of these proving nonhemolytic and indol-producing. Four of the 5 hemolytic colonies grew in subculture and 3 of them were indol-negative. The 4th, which was indol-positive, was again plated out and shown to be a mixture; 20 colonies of each type, hemolytic and nonhemolytic, were picked, and all the hemolytic failed to produce indol, while the nonhemolytic cultures were indol-positive. In every instance, except the 4 recorded above, pure hemolytic strains failed to produce indol. It seems probable, therefore, that the low proportion of indol formers among our hemolytic strains is due to the rigorous isolation method we have employed.

We have tested 35 strains for saccharose fermentation and production of gas in dextrose agar with results almost similar to those recorded by Stillman and Bourn (29 strains). The larger proportion of the hemolytic strains fell into group C (table 5), (saccharose +, indol —, gas in dextrose —), 54% of our strains having these characters, as compared with 79% of the Stillman and Bourn strains. This contrasts with 11% of nonhemolytic strains in this group as observed by Stillman and Bourn and 6% by Jordan and Reith. The A group (indol +, saccharose —, gas in dextrose —) which is the largest among the nonhemolytic strains (Stillman and Bourn 52%, Jordan and Reith 35%) found no representative among the hemolytic Pfeiffer bacilli.

The frequency of occurrence of the hemolytic bacillus in 228 healthy persons (15%) and in 85 persons suffering from colds (16%) was about the same. These figures do not indicate that the hemolytic bacilli are especially abundant in the throats of persons suffering from colds. Our total percentage of positive findings of hemolytic bacilli in healthy persons is considerably higher than that reported by Pritchett and Stillman, who found "Bacillus X" in about two dozen persons during a series of cultures made from several hundred healthy people. It is, however, lower (15%) than the figures given by Rivers for October, 1919 (30%), or those by Bloomfield for August, 1921 (33%). The figures of the latter, for an earlier period, are not readily comparable with our data since his tabulation shows that cultures were obtained several times from the same person. Kristensen (1922) found hemolytic bacilli in 16 of 200 persons.

A few unpublished experiments performed by Hudson in this laboratory indicate that the hemolytic strains have a lower pathogenicity

for mice than the nonhemolytic,[28] although mice sometimes die when injected with this organism. Pritchett and Stillman were unable to demonstrate any pathogenicity for mice, rats or rabbits.

Both hemolytic and nonhemolytic types may occur in the same person. Of 34 cases in the series already described in which hemolytic bacilli were found in healthy persons, nonhemolytic strains were also isolated from the same plate in 6. A particularly interesting instance of the similarity of bacteriologic flora in persons who are in more or less intimate contact with one another was encountered in 1922 in a group of healthy school children (7-8 years old) in whom throat swabbings were being made for control purposes.

TABLE 10

INCIDENCE OF PFEIFFER BACILLI IN 10 NORMAL THROATS DURING 6 WEEKS.

Case	First Swabbing 4/19/22	Second Swabbing 5/3/22	Third Swabbing 6/2/22
21	H* and N	H and N	H and N
22	H	H and N	H
23	H	H	N
24	H and N	N	H
25	N	H and N	N
26	H	N	H and N
27	H	H and N	N
28	H and N	H and N	H
29	H and N	H and N	No Pfeiffers
30	Not swabbed	H	H and N

* H = hemolytic; N = nonhemolytic.

In contrast to this was a similar series of throat swabbings of 9 school children with sore throats made between April 7 and April 26, 1922. No Pfeiffer bacillus colonies were detected at any time on the plates made from 4 throats; in 1 the nonhemolytic Pfeiffer bacillus was present in all 3 throat examinations; in 1 precent in 2 examinations, absent in 1; in 1 present once, absent once; in 1 both the hemolytic and nonhemolytic types were present in one examination and in 1 both were absent. This is in line with what one of us [20] has pointed out previously, that under certain conditions a group of persons in contact with one another may harbor similar assemblages of micro-organisms without its being necessary to conclude that these organisms have any pathologic significance.

Our data on the seasonal distribution of the hemolytic bacillus are so limited that conclusions are hardly warranted. It may not be altogether a matter of chance, however, that the hemolytic Pfeiffer

[28] Jour. Infect. Dis., 1922, 30, p. 433.

bacillus was almost entirely absent from 105 throat swabs made in the warm months of the year (May, 1; June, 2; July, 0; August, 0) although in the same months the nonhemolytic Pfeiffer bacillus was found 35 times in the same 105 persons. In March, on the other hand, the hemolytic type was found 9 times in 30 examinations and in November 10 times in 30. Bloomfield's observations [26] seem to show a lower proportion of positive findings in August than for the period November-May, although his tables are difficult to interpret, owing to the inclusion in one summary of repeated examinations of the same persons with single examinations of others. Table 9 emphasizes the caution that must be used in regarding throat swabbings of different groups as typical either of locality, season or disease incidence.

ANIMAL STRAINS

Throat swabs from a number of laboratory animals have been made in the usual way on blood-agar and oleate medium plates. The examination included 48 rabbits (34 healthy, 14 with snuffles), 14 guinea-pigs, 6 dogs, 3 sheep, 1 goat. Two strains of hemophilic gram-negative bacilli morphologically similar to the Pfeiffer bacillus and showing the satellite phenomenon have been isolated from rabbits. One rabbit (R 24) had been in the laboratory for about 9 months and had been used for producing B. paratyphosus A serum; the other animal (R 28) was a recent purchase. Both were apparently quite healthy, and the respiratory tract seemed normal. Isolations were made from nasal swabs on oleate medium.

One strain (R 24) was hemolytic, did not reduce nitrate, produce indol or ferment dextrose or saccharose, thus departing in the matter of nitrate reduction from the Pfeiffer bacillus type. The other (R 28) was a typical non-hemolytic Pfeiffer bacillus, reducing nitrate, fermenting dextrose with gas production, forming acid in saccharose, but not producing indol. It could not be distinguished culturally or morphologically from the rest of our Pfeiffer stock.

Kristensen [3] has reported finding organisms in 5 of 60 guinea-pigs which, according to all his tests, must be classed as Pfeiffer bacilli. Nitrate reduction was apparently not determined. He found no hemophilic bacilli in 43 mice or 33 horses.

SUMMARY

The most common type of cell in cultures of the Pfeiffer bacillus from various sources is the very small, short, thick rod with rounded

ends. The morphologic variants showing differences in cell size and cell grouping, and particularly the filamentous forms, are all closely connected with and may proceed from the more typical forms. Morphology is less affected by particular nutrient substances or by hydrogen-ion concentration than sometimes supposed. The tendency to produce filamentous forms or to perpetuate certain cell groupings seems to be more strongly inherent in certain strains than in others.

Indol was produced by 57% of 220 strains from a great variety of sources and locations. Indol producers were found in somewhat higher proportions among the strains from colds and other pathologic conditions than from normal throats.

Nitrate reduction was confirmed as an important differential character of these organisms, all of the 287 strains tested being positive as contrasted with the negative results with all B. pertussis and B. bronchisepticus strains.

Fermentation tests (dextrose, saccharose) permit the separation of the Pfeiffer bacilli into several groups, but these groups are not characterized by agglutinative homogeneity and seem to have no epidemiologic significance.

Pfeiffer bacilli were present in a somewhat larger proportion of persons who had had influenza than in those who gave no history of this infection. They were also found more commonly in the throats of persons with colds than in those of healthy persons. A limited number of examinations showed them to be decidedly more common in the throats of healthy persons in March, 1922, when clinical influenza was prevalent than at other times of the year. Pfeiffer bacilli were found much more commonly and abundantly in the throats of some healthy persons than in those of others.

Hemolytic Pfeiffer bacilli have shown two types of hemolysis, each type breeding true, but otherwise not culturally separable. Filamentous forms were found more frequently in cultures of the hemolytic strains than in those of the ordinary nonhemolytic strains. Slight differences in colony characters and in biochemical qualities place the hemolytic strains somewhat apart from the nonhemolytic types. The hemolytic Pfeiffer bacilli do not retain their vitality on the ordinary culture mediums as well as the nonhemolytic. The hemolytic bacilli were found in practically the same proportion in persons with colds (16%) and in healthy persons (15%); they may occur in a large proportion of a particular group of healthy schoolchildren (table 9). In a limited series

of observations they were found much less abundantly in the summer months than in the winter.

A bacillus resembling in every respect our stock of typical non-hemolytic Pfeiffer bacilli was isolated from the respiratory tract of a healthy rabbit.

TRANSMISSIBLE LYSIS OF BACILLUS PYOCYANEUS

THREE PLATES

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PRIMARY OBSERVATIONS

During an attempt to obtain a fresh culture of B. pyocyaneus for class purposes, cultures were made from green pus taken from a suppurating lesion on the forehead of a private patient. This aspect of the work was carried out through the courtesy of Dr. Sleight of Battle Creek. In these cultures no colonies recognized as pyocyaneus were observed. The sample of pus was laid aside and a fresh sample obtained from the same case. Plates poured from the second sample gave, among colonies of staphylococcus, a small number of colonies showing the characteristic growth and blue-green pigment of B. pyocyaneus.

At the outset nothing peculiar was observed in the appearance of the agar slant cultures. After 48 hours' incubation, however, the tubes showed a striking appearance. The color was the typical blue-green of pyocyaneus and extended deep into the medium; the growth was profuse, spreading, and presented the usual glistening luster. The feature which immediately caught the eye, however, was the presence of pitted or pocketed areas, more or less circular, having a diameter of from 1 to 4 mm., appearing on the surface of the bacterial growth and apparently extending down to the agar substratum.

In these areas the normal culture had largely or completely disappeared, so that the floor of the pockets was depressed and bordered by nearly perpendicular walls comprising the mass of normal culture. By strong transmitted light these pockets appeared as transparent areas against the blue-green culture. If, however, the slant agar tubes were so held that the corroded areas reflected the light, they presented a marked iridescent effect, throwing glints of bronze with its components of yellow, green and red; or sometimes distinctly blue. The smaller areas were more likely to appear as silvery flecks.

The number of such areas on the agar slant cultures varied from 1 to 14. Usually they were discrete, but if the number was large, they became confluent. After they were first observed, the areas

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increased slightly in size at the expense of the surrounding culture, but seldom did they exceed a diameter of 4 mm. Continued incubation at 30 C. did not serve to increase their size; and, with the aging of the cultures standing at room temperature, they became progressively less distinct.

Subcultures from these agar slants in which the pockets first appeared perpetuated the same characteristics, frequently with a greatly increased number of areas, up to 30 or 40. In some cases the number was sufficiently great to cause, through their confluence, an almost complete disappearance of culture within a period of from 3 to 4 days. Over the surface of such agar slants, from which the culture had largely disappeared, one could detect by means of the needle only shreds of viscous growth.

It was not only from the region of the pockets or from the culture immediately surrounding these pockets, however, that it was possible to obtain in subculture on agar a duplication of this phenomenon; when the needle was touched to what were manifestly regions of normal culture and then streaked on agar, a similar pocketed growth was secured.

Whether cultivations in infusion broth (P_H 7.8) were made from the pockets or from the areas between, the results were the same. At 37 C. the growth appeared on the surface in the course of from 8 to 10 hours, and a little later formed a tenacious membranous pellicle from the under side of which streamers of growth would fall by gravity slowly down through the tube. On agitation the pellicle formed a viscous aggregate, even to the extent of ropiness, which eventually would sink to the bottom of the tube as a highly viscous sediment. At the end of a week to 10 days the entire body of broth possessed a syrupy or almost gelatinous consistency. The medium usually showed a yellow-green color by the end of from 16 to 18 hours, and after a day or two the surface layer took on the typical blue-green color characteristic of pyocyaneus. This green layer would extend progressively down through the tube until the entire tube was a homogeneous blue-green color. In its final stage of oxidation, the color became nearly black.

To any one familiar with the appearance of the areas of erosion found in slant cultures containing the lytic principle or bacteriophage of d'Herelle,[1] the phenomenon just described could not fail to suggest the possibility of the participation of a lytic agent in the production of the pocketed areas. This paper deals rather broadly with an exami-

[1] The Bacteriophage: Its Rôle in Immunity, 1922.

nation of the phenomenon in relation to its probable cause and extended effects. A later paper will consider in greater detail some specific features of the subject which here are touched on briefly.

STUDY OF THE PRIMARY CULTURE

Division of the Original Strain into Two Components.—The original culture was plated on infusion agar (P_H 8). After incubation at 37 C. for 12 hours the plates stood at room temperature. At the end of incubation, colonies appeared which resembled each other closely. They were yellowish green and surrounded by a narrow zone of diffusing yellow-green pigment. Close examination of the colonies at this time revealed a few on the surface of which could be seen minute silvery flakes, sometimes one only, sometimes several on a single colony. Within the following 12 to 24 hours these colonies became further differentiated from those not possessing the silvery patches. The size of these areas increased, and they visibly penetrated the culture mass, eventually forming roughly circular, bronze or iridescent pockets similar to those observed earlier on the agar slants. These crater-like pockets attained their greatest size when the colonies were about from 4 to 5 days old, and after this time usually underwent no further change. Although the size of the pockets on the colony surface varied from 0.5 to 4 mm., they were roughly proportional to the size of the colony. Well isolated colonies (2 or 3 per plate) having a diameter of from 25 to 35 mm. would have pockets of the greatest size, while the small colonies (crowded plates), having a diameter of only 6 to 10 mm., would have pockets only 1 to 2 mm. in diameter. In the smaller colonies, however, the small lytic areas frequently became confluent and destroyed a great part, or sometimes all, of the colony mass.

Many colonies, instead of showing the craters on the colony surface, after 24 hours' growth manifested a silver patch or a short silver arc on the outer border of the colony. Usually this arc would extend circumferentially to involve 60, 90, or even 180 degrees of the colony border. Colonies were frequently observed possessing a complete silver halo, but in such cases the halo appeared to arise from several marginal points rather than to spread around the colony from a single point. The tendency was clear for such silvery arcs or halos to extend backward into the colony, leaving, as a result of the corrosion, either a diminished, but still circular, colony mass, or irregular islands of normal culture indented by bays and gulfs of corroded surface. In some cases the normal colony mass was completely destroyed by the rapid

invasion of the silvery areas, which eventually took on the bronze or iridescent color previously mentioned. It was observed in plating that the surface colonies that started at the surface of the agar more commonly possessed only the circular pockets inside the colony, or a short marginal corrosion, while colonies which emerged on the surface after a development of 24 hours or more deeper in the medium, showed entire circumferential corrosion with the concomitant halo formation almost from the start. Colonies of this kind were rapidly destroyed, usually before they attained a diameter of 4 mm. Apparently the lysis had started in the young colony beneath the surface of the agar.

At the beginning of the work about one-half of the colonies on the plate showed more or less corrosion by the end of 48 hours. The remainder of the colonies approximated more closely the "normal" pyocyaneus type but showed certain marked differences from both a "normal" colony and from the corroded colonies mentioned above. In the first place, they revealed a less expansive but a more massive growth, with a tendency to convexity, such as is so common in colonies of B. aerogenes and of Friedländer's bacillus. They showed smooth entire margins, while the margins of the corroded colonies were more irregular, fimbriated and leaf-like. The consistency of these homogeneous colonies was markedly viscous.

But perhaps the most striking difference between the homogeneous and the corroded colonies was in the production of pigment. While the latter were active producers of the blue-green pigment (pyocyanin), the former produced only the yellow-green pigment (fluorescin), and were sometimes nearly without color. While the colonies showing corrosion manifested progressive disintegration throughout the period of colony growth, the homogeneous colonies underwent no further change except a gradual increase in their viscosity. Colonies which did not show areas of corrosion within the first 48 hours of growth usually did not show them at all.

Microscopic examination of the corroded colonies under no. 3 objective showed in the corroded areas disintegrating bacterial cells together with large aggregates of needle-like crystals. Smaller aggregates appeared also in portions of the colonies where microscopic examination of the surface failed to reveal evidence of corrosion. These needle-like crystals also appeared in great number through the agar surrounding the colony and sometimes several centimeters from it. These crystalline deposits will be referred to subsequently.

Subcultivations on slant agar were made from the two types of colony. The colonies lacking the corrosions yielded a homogeneous growth with slight yellow-green pigment production or none at all. The surface showed a moist, glistening luster. After 48 hours' growth the consistency was highly viscous. After a week's growth at room temperature the growth at the bottom of the tube frequently became definitely pink. Eventually both the culture mass and the substratum became brownish or brownish red; sometimes a brilliant red. Subcultivations from the corroded colonies to slanted agar gave an appearance similar to that observed in the original slant agar tubes, namely, a thin, expansive growth studded with areas of corrosion and yielding a brilliant blue-green pigment which progressively penetrated the substratum. In the course of time the color changed to greenish black.

Colonies of the corroded and the homogeneous types were submitted to further plating. The homogeneous colonies gave rise only to homogeneous colonies, while the corroded colonies gave both homogeneous and corroded colonies as in the first plating test. Plating out through continued series always gave the same result.

The results of the work up to this point were sufficient to indicate that the observed phenomenon involved a destruction of the bacteria by progressive lysis; and, moreover, that this lysis failed to take place in a certain proportion of the organisms. In view of this circumstance, for the purposes of the following discussion, the strain of pyocyaneus originating from the corroded colonies will be henceforth referred to as the lytic or lysogenic strain or the L strain, while the culture derived from the homogeneous colonies will be referred to as the resistant, the nonlytic or the R strain. In dealing with the subject of transmissible lysis in the case of the d'Herelle principle acting on other organisms, use has frequently been made of the terms R strain and S strain to indicate respectively "resistant" and "sensitive" cultures. In the present case, for reasons which will subsequently appear, I desire to reserve the use of the term "S strain" for a type of culture which will be described later.

Further Study of the "L" Strain.—Artificial Colonies: In order to be able to observe more clearly the nature of the colony development of the lytic strain, the following procedure was employed: The culture was plated by the usual means. As soon as it was possible to recognize a colony of the lytic type by observing the first silvery fleck on its margin or surface, this colony was touched with the needle, which was in turn touched to the surface of the medium in the center of a

freshly poured, cooled, agar plate. The plate was incubated at 37 C. The growth of such an artificial colony began at once, and in the course of about 5 to 8 days such colonies attained a diameter of 50 to 65 mm.

After the first 12 hours of growth of such a colony it appears as a grayish green placque with a more or less fimbriated margin and has a diameter of 10 to 18 mm. It may be surrounded by a narrow zone of yellow-green. At the center may be observed an area of lysis composed of a number of small lytic pockets which have become confluent. Outside of these lies a zone of apparently normal culture. As growth proceeds, this area increases in size and, in addition, silvery flecks appear on the surface of the colony in areas which were previously homogeneous. These lytic flecks increase in size and depth, becoming more round, until they evolve, with further growth of the colony, into circular pockets having a diameter of 2 to 4 mm. By reflected light they present a beautiful iridescence or a bronze sheen, producing a picture which it is difficult to describe. In these areas of lysis the culture mass has largely disappeared, and the surface appears depressed to the level of the substratum.

When these lytic pockets are examined under no. 3 objective they are observed to be quite free from at least observable bacterial masses and contain numerous needle-like crystals which for the most part lie free, but which are often grouped into starlike or radial aggregates of considerable size. Even when the needles lie free they tend to be oriented radially to the lytic area, and may be seen to extend into the normal culture mass which forms the wall of the lytic pocket.

With the further growth of the colony, the lytic areas continue to come in at irregular locations, but always appearing nearer the free edge of the growing colony. In rare cases they may appear on the margin and extend along the border to the extent of a considerable portion of the circumference. Sometimes this marginal, lytic arc determines a termination of growth at this point, while the culture continues to grow beyond it at either side; such a condition serves to produce the appearance of marginal invaginations, or wedge shaped excavations, into the colony. In other cases the marginal, lytic arc does not cause a termination of growth of the colony at this point, but the culture, appearing to "recover," transcends this barrier and proceeds normally beyond it. In such cases the lytic arc comes to lie as a concentric lytic band within the colony.

Number of Lytic Areas: The number of particulate lytic areas in the large colonies of pyocyaneus varied considerably. In some colonies

having a diameter of 50 to 60 mm. there might develop 200 or more, with marked coalescence due to crowding. In other colonies of the same size, not more than 2 or 3 such lytic areas might appear, removed from the central lytic area, or from each other, by 15 to 20 mm. of "normal" culture. It could not be observed that the size of these areas was appreciably greater because of their isolation; about 3 mm. was the average size, whether crowded or discrete. Sometimes, however, the 50 to 60 mm. colonies, showing not more than 2 or 3 lytic areas, would suddenly develop at the periphery a nest of silver flecks, which would proceed to determine an extensive marginal lysis, producing the "silver edge" colonies observed more commonly in direct plating of the L strain.

Abortive Lytic Areas: It sometimes happens, however, that these particulate lytic areas do not undergo development to their normal limit, but suddenly cease from their erosive action and become overgrown by the surrounding culture. This is most likely to happen in colonies in which the number of areas is small. When such termination of the lytic area occurs, although the pocket becomes filled with fresh bacteria, the old outline of the crater continues to be apparent, and appears as a ring by transmitted light. This phenomenon is duplicated in kind on slant cultures, when small lytic pits may appear for a day or two, only to diminish in size and to become filled in by the increasing bacterial mass. If these pits are numerous, they present to the surface of the culture a coarsely granular appearance which is eventually superceded by the more common glistening luster. Indeed there exist in the slant cultures all degrees of macroscopic manifestation of lytic action with similar variations in its permanence. In general it may be said, however, that when a lytic area has been formed in a colony, involving an erosion down to the agar substratum, this area does not become overgrown but remains apparently denuded of bacterial growth. As we shall see, however, some bacteria usually remain on the floor of the pocket, and these organisms give rise to an R strain.

In large artificial colonies of the L strain (those attaining a diameter of 60 mm. or more), although the lytic areas may become confluent, the entire culture mass of the colony seldom completely disappears as a result of extension and coalescence of the erosive areas; the main culture mass, for a time at least, remains homogeneous. After a week or 10 days the colony has attained its maximum size and commences to degenerate. The brilliant blue-green of the colony and its surrounding medium becomes dulled, turning later to a brownish green, then

brown or even brownish black. As a result of drying, the culture mass flattens and hardens although bronze areas still mark the points of lytic action.

Degeneration of the Colony by Mass Lysis: Although, as stated above, the large colonies are not commonly completely destroyed as a result of the extension and coalescence of the distinct lytic areas, the colony mass may undergo a sort of retrogression which approximates lysis in its final result. Such retrogression originates in the central portions of the colony and is synchronous with the rapid extension of the colony at the periphery. This retrogression, occurring in mass rather than in particulate areas, expresses itself by a perfectly graded change in the optical density of the culture, deminishing in effect in passing from the center of the colony to the periphery. Against these broad areas of mass lysis the particulate lytic areas, which originated earlier, still maintain their integrity. It is clear, however, that in the regions of mass lysis the change is not due entirely to a diminished physical density of the remaining culture mass. This does not disappear, but takes on first a translucency and later a gelatin-like transparency, so that a line of type may easily be read through a 4 mm. layer of agar and its culture layer.

The appearance of the areas of mass lysis differs from those of particulate lysis. They show no bronzing of the surface, and one can still perceive many bacterial bodies in the transparent mass. When these areas of mass lysis, which have their beginning in the central regions of the colony and progressively destroy the growth that has not already been destroyed by the particulate areas (constant incubation), or which have their beginning in the zones where the particulate areas have finished their work (intermittent incubation), are examined under no. 3 objective, the following picture appears: The field is studded with innumerable minute lytic centers, at the middle of each of which lies a small aggregate of needle-like crystals. These areas vary in size from 5 to 50μ and tend to be circular. Crystals cannot be observed in the smallest areas. These minute lytic centers make no showing on the surface of the medium. Inside of them the bacteria seem to have largely disappeared. They are present in greatest number where the bacterial mass is most transparent, diminish in number where it is merely translucent, and disappear near the fresh borders of the colony where the bacterial mass is still undergrowing growth. All gradations in number of minute lytic areas are found, and these are directly proportional to the density and age of the culture mass.

The final stage in the retrogression of the large colonies of the L strain may therefore present a variety of pictures. The most common is that of partial destruction of the colony by the large particulate lytic areas, followed by destruction of the remainder of the culture by mass lysis. This in turn is followed by cessation of growth at the periphery and the drying of the marginal remainder of unlysed culture. In other cases, however, marginal lytic arcs may intervene and not only terminate further peripheral growth, but also initiate a progressive lysis toward the interior of the colony, cleaning away the bacterial mass as lysis proceeds slowly toward the center, where irregularly shaped islands mark the remnants of the colony. The conditions of growth which favor lysis by particulate areas or favor mass lysis are not yet recognized.

Microscopic preparations were made from the areas of particulate lysis and from the areas of mass lysis and examined by dark-field illumination and in stained films. The dark-field examination revealed a small number of bacteria which were rather shorter and plumper than the normal type. A few still possessed motility. With the bacteria were a vast number of minute granules, for the most part circular or oval, and measuring probably 0.02 to 0.05 of a micron. In preparations stained with the common dyes and with the Romanowsky stain a few bacteria were observed surrounded by masses of degenerating cells. In the field stained with Romanowsky stain were present great numbers of small granules staining pink, and perhaps comparable with those seen by dark-field examination.

Other Characteristics of the L Strain.—Growth Energy: A point of considerable interest in relation to the artificial colonies of the L strain is their remarkable "growth energy." Two sterile agar plates are prepared with sufficient medium to give a depth of at least 4 mm. One plate is inoculated by stabbing in the center with the L strain, the other plate with the R strain. The plates are inverted and incubated at 37 C. for 24 hours, then kept at room temperature. At the end of 12 hours the L strain colony has a diameter of 18 mm., and the R strain colony has a diameter of 6 mm. After about 10 days the R colony has reached its maximum size of 20 mm., while the L colony has attained a diameter of 50 to 60 mm., and may continue to grow for several days longer to attain an observed maximum of 65 mm. At the end of the growth period the R colony is homogeneous in texture and colored

a yellowish brown against a bright yellow background; the L colony is pocketed and colored a brilliant blue-green against a dark green background.

Chameleon Phenomenon: With further reference to the colonies of the L strain, it may be added that these were the only ones to present the so-called "chameleon phenomenon" first reported by P. Ernst [2] (1887) and later by Jordan [3] (1899). This phenomenon was observed on potato cultures of pyocyaneus, and involves a rapid change in color of the surface of the bacterial mass when it is disturbed by touching with the needle. A play of colors is seen over the lacerated area within a few seconds, probably due to the rapid oxidation of the basal pigment substance to pyocyanin. In the present instance the phenomenon was observed, not only in potato cultures, but in colonies of the L strain on the agar plate. The colonies which gave the best reaction occurred in plates which had not been recently opened, and were the relatively few colonies which, after several days' growth, still presented the faintest pigment production. Indeed some were nearly white, but they quickly became green in the lacerated portions.

Deposition of Crystalline Bodies: Another interesting feature relating to colonies of the L strain is the deposition of crystals in the surrounding agar. It has frequently been observed that crystalline bodies appear in agar where it begins to dry. But it is by no means common to find crystalline bodies in a fresh agar on which a culture is undergoing rapid growth. Attention was first called to this phenomenon by the report of Dorset [4] in 1897. In the present work, these crystals were not produced by the R strain, but by the L and the S strains, notably in the former. They occurred, not only in the bacterial mass, but also in the agar medium surrounding the colonies, sometimes removed from the colonies by 40 to 50 mm. In some cases the crystalline aggregates were of such size as to be visible to the naked eye. They appeared to be composed, for the most part, of pyocyanin and fluorescein.

Relation of Temperature to Appearance of Lytic Areas: When plates bearing a single colony of the L strain are kept under constant incubation at 37 C., the distribution of the particulate lytic areas is usually irregular; they appear to have a "chance distribution." It was observed, however, that when plate colonies were submitted to inter-

[2] Ztschr. f. Hyg. u. Infektionskrankh., 1887, 2, p. 369.
[3] Jour. Exper. Med., 1899, 4, p. 627.
[4] Centralbl. f. Bakteriol., I. O., 1897, 21, p. 473.

mittent incubation, each period followed by a period of 8 to 10 hours at room temperature, the colonies presented a different appearance. Under these conditions the particulate lytic areas occurred in successive concentric zones surrounding the initial lytic area in the center of the colony. The interzonal spaces comprised homogeneous unlysed culture. The influence of incubation on the development of zones containing the particulate lytic areas appeared to be something as follows: A colony of 8 mm. containing a small central lytic area, surrounded by a zone of room-temperature growth containing no lytic areas, is incubated over night. The next morning a new growth is observed measuring 3 mm., measured on any radius, thus increasing the diameter of the colony to 14 mm. This new growth is thin and delicate and shows no lytic areas —or at most a few silvery flecks. The plate is left at room temperature during the day, and during this time numerous lytic areas appear in the night's growth. These in time increase in size to a diameter of 2 to 3 mm. In the meantime, the growth has been extending slightly at room temperature. At night the plate is returned to the incubator and removed the following morning. During the second day lytic areas appear in the growth of the preceding night, but not in the growth of the preceding day. In this manner rings or zones of lysis occur in successive stages; and between them lie zones in which particulate lytic areas are, for the most part, absent. In general it may be said that growth at 30 to 37 C. markedly favors the lytic action.

To summarize: In the progressive lysis of the pyocyaneus colony two factors are involved: (1) the large depressed, particulate lytic areas which accomplish the destruction of culture with relative rapidity; (2) the mass lysis, accomplished through the agency of a vast number of minute lytic centers, working below the surface of the growth, expanding only within narrow limits, but frequently accomplishing, in the long run, more extensive lysis than is effected by the large surface erosions. Thus all colony lysis of pyocyaneus is, in reality, particulate lysis (it originates from lytic centers), the difference in the ultimate effects being due merely to variations in the depth, size and relative frequency of the lytic centers from which the lytic action originates. The mass lysis, it may be noted, is the form of lysis in pyocyaneus which most closely resembles what presumably happens in the lysis of Shiga mass cultures on agar. The difference is probably only one of speed or energy of reaction.

Subcultivations from Different Parts of the Lytic Colonies: It has been stated that subcultures from the large lytic areas on slant agar

gave lytic growth. When the needle was touched to areas of "normal" growth in the same culture and streaked on agar, a similar lytic growth was obtained. In other words, subcultures from any portion of a colony which showed the least evidence of lysis never failed to produce a lytic growth; and the cultivation from lytic areas of a lytic colony gave growth that was no more "lytic" than cultivation from "nonlytic" areas of the same colony. The lytic agent was manifestly present in all parts of the colony, but came to expression only in certain centers.

In all these agar slant subcultures of the L strain the following point was noteworthy: The lytic areas seldom developed on the upper part of the slant growth; they were limited to the middle and lower areas. This point will be referred to subsequently.

Transmission of Lysis to Nonlysed Culture and to Other Colonies: Areas of lysis were touched with the needle and then transferred to other areas of the same colony where lysis was not apparent. Particulate lytic areas did not develop at these points, and no change in the growth was observed. In a similar manner the needle carrying lytic culture was touched to the border and centers of nonlysing colonies. These colonies subsequently failed to show evidence of lysis, although the lysing strain might grow out from their margin where the needle also touched the surface of the agar. This was rather to be expected since it was known that these nonlysing colonies already harbored the lytic agent.

In this connection may be noted the "accidental" lysis of colonies of other bacterial species. On the Petri dishes carrying the artificial colonies of the lytic strain colony contaminations from air bacteria sometimes occurred. Sometimes these colonies had a position not far from the rapidly extending border of the lytic colony, which would then overrun and engulf them. In two cases the accidental colonies (which were found to comprise small, nonchromogenic rods) had attained a diameter of about 4 mm. when the edge of the lytic colony reached them and began to circumvent them. Growth of the accidental colonies then ceased, and the bacterial mass began to disappear, leaving an apparently bare surface. Over this surface the pyocyaneus colony grew from two sides to the extent of about one-half the area, and then continued to extend beyond it by a centimeter or more.

Another case was of special interest. In the center of a Petri dish a nonlytic colony (carrying the lytic agent) had attained a diameter of about 15 mm. A contaminating aerogenes-like colony of about 12 mm. was developing a short distance away, so that a space of 10 mm.

separated the two colonies. The edge of the contaminating colony approximating the nonlytic colony began to undergo lysis, and eventually the whole colony was more or less affected. Three subculture streaks were made from the lysing colony across fresh agar surface. A heavy glistening growth occurred within 12 hours; lysis then started in the center of the streaks, and within 24 hours more had destroyed a large part of the bacterial mass. Unfortunately, no control culture was taken from the original colony before lysis occurred. The nature of these colonies has not yet been ascertained, and it is a question as to whether they were destroyed by the "pyocyanase" or by the lytic agent or both.

Further Propagation of the L and R Strain.—Other Biochemical Features: The original lytic colonies were perpetuated for 40 generations on slanted agar, the transfers being made at intervals of from 3 days to 2 weeks. For the first 28 transfers the brilliant green pigment and the lytic areas were maintained in their original integrity. After this point, however, the pigment began to diminish in amount and intensity, and the lytic areas became less and less numerous, until by the 36th transfer both pigment and lytic areas had disappeared from the culture which now produced only a yellow-green pigment. In other words, during these transfers, the lytic strain came to resemble the nonlytic strain which had been carried in a parallel series. In the 40th generation, the two strains could not be told apart macroscopically. At this time the original characteristics of the lytic strain could still be obtained in subculture from any of the early tubes of the lytic series.

The rapidity with which the lytic and the apparently correlated chromogenic features of the L strain were lost in transfers on agar was found to be influenced by the mode of cultivating: If the inoculum for each successive tube of agar were taken from the upper quarter of the former slant, the characteristic features were lost quickly (18 to 20 transfers); but if the inoculum were taken from the lower portion of the tube, where lysis was more in evidence, the loss was less rapidly effected. In the series mentioned above, the transfers were made from about the middle of the tube. This result is due to the circumstance that in slant cultures the majority of the lytic pockets arise near the middle of the slant. They also arise below the middle if condensation water is not present, but seldom do they appear in the upper third or quarter of the slant, although the bacterial growth here is by no means diminished.

In infusion broth (P_H 7.4) no difference except in pigment production was observed between the R and the L strains. Both grew

with great luxuriance, forming a heavy membranous pellicle from the under side of which the growth fell progressively to the bottom of the tube, forming in both cultures a viscous precipitate. After a few days the entire broth became viscous. While the color of the broth cultures of the R strain remained yellow-green, the tubes of the L strain began to show the typical blue-green at the top of the tube after about 2 or 3 days; and, if the tubes were left undisturbed, this zone would extend toward the bottom of the tube, giving a deep blue-green tone which eventually changed to greenish black, or sometimes to brownish black. The R strain failed to develop such pigment, and even in the L strain the power to form pyocyanin was diminished through successive transfers in plain infusion broth.

The R strain which had lost its ability to form pyocyanin was run through a series of 6 infusion broth tubes containing 5% glycerol, with the result that the pyocyanigenic power returned to a slight extent, as indicated by shaking with chloroform, also by growth on agar slants. The amount of pyocyanin was not sufficient, however, to produce blue-green broth cultures.

The L and the R strain were inoculated into milk containing brom-cresol-purple as indicator. Neither strain caused a definite coagulation, although both rendered the milk slimy after some weeks. After about 2 weeks the L strain produced a marked reddening, together with translucency through the disappearance of the casein. The R strain caused the same changes, but only after about 5 weeks. Subcultivations from milk to slant agar at the end of 5 weeks showed that the L strain was still pyocyanigenic, while pyocyanigen production had not been brought back to the R strain. The only other strains which produced rapid reddening and translucence in milk were a Buffalo strain, which became lytic during the course of study, and a Wisconsin strain, representing a nonlysogenic, sensitive type.

The strains were also inoculated into gelatin tubes. The L strain produced liquefaction of 9 cm. in 3 to 4 days. The R strain at first liquefied slowly; later, not at all. Various R colonies varied in their original liquefying ability.

STUDY OF CULTURE FILTRATES

Filtrates from the L Strain.—Forty c c. of broth were inoculated with the lytic culture, incubated at 37 C. for 24 hours, and filtered through a Berkefeld candle. At the time of filtration an inoculation was made to slant agar; typical blue-green lytic growth appeared. The

filtrate was incubated to test for sterility. To a tube of 1 c c. of filtrate, about 9 c c. of broth were added, and the tube inoculated with one loop of a young broth culture of the L strain. Growth equal to that of the control resulted. The same procedure was repeated 9 times with 9 intervening filtrations and "feedings," and the result was invariably the same—the filtrate always failed to inhibit the growth of the L strain.

A new start was made, and the L strain filtrate was not diluted with broth, but used in full strength, and inoculated with smaller amounts of the lytic culture. Six tests were made, with intervening filtrations and feedings, and the results were the same—no inhibition of growth of the L strain resulted. The filtrate was also tested against the R strain, but here also there was no inhibition.

Another filtrate was prepared from some old tubes of the L strain which had been standing in the laboratory for about 5 months. This was divided among 4 tubes, which were inoculated with 1 loop of young broth culture of (1) L strain, (2) R strain, (3) a Parke-Davis culture, and (4) a Mulford culture. Tubes 1 and 2 gave no inhibition; 3 gave slight inhibition, and 4 gave marked inhibition.

Another filtrate was prepared from a 24-hour culture of the L strain and the same test applied, with the same cultures and with corresponding results: Of the 4 cultures tested, only one (Mulford) was sensitive to the action of the filtrate from the L strain. Accordingly, a titration of the inhibition power for the Mulford strain was performed. Three 8 c c. tubes of broth were set up. To (1) was added 5 drops of the L filtrate, to (2) was added 15 drops, and to (3) no filtrate was added. To all tubes was added 1 loop of an 8 c c. broth tube containing 1 loop of Mulford culture. After 12 hours (1) showed a haze, (2) was negative, and (3) showed fair clouding. After 36 hours all showed grade 3 clouding.* The filtrate thus showed some growth-inhibitive power, but very weak. An attempt was made to increase it by the feeding method.

The L Filtrate Enhanced by Mulford Feeding: To about 30 c c. of this filtrate were added, on 2 successive days, 40 drops of 12-hour Mulford broth culture; incubated, then filtered. Sixty drops more Mulford culture were added. The culture was again filtered and tested for inhibition power, the inoculations of all 8 c c. tubes being 1 loop of 1 loop of the respective culture in 8 c c. of broth.

The tabulation shows increased inhibition for the Mulford culture, but slight for the Parke-Davis. The filtrate again received a "feeding"

* In the present records (+ + + +) means maximum turbidity; (+) first visible clouding; (+ +) and (+ + +) intermediate grades of clouding.

with fresh Mulford culture and was again tested; and this process was repeated for 22 successive titrations. The detailed results do not require presentation, except in the final instance representing the maximum titer of the filtrate against Mulford and against several other strains of pyocyaneus. In all the following cases, the 8 c c. broth tubes, including

TABLE 1

TITRATION RESULTS OF A MULFORD FED L FILTRATE AGAINST MULFORD AND PARKE-DAVIS CULTURES

Filtrate	Mulford Culture			Parke-Davis Culture		
	(1) 2 Drops	(2) 5 Drops	(3) Control	(1) 2 Drops	(2) 5 Drops	(3) Control
After 12 hours	—	—	++	++	+	+++
After 24 hours	—	—	+++	++++	+++	++++
After 48 hours	++	+	++++	++++	++++	++++

TABLE 2

TITRATION RESULTS OF INHIBITIVE POWER OF A MULFORD FED L STRAIN FILTRATE AGAINST MULFORD

Time of Observation	Dilution of the Lytic Filtrate				
	200,000	20,000	2,000	200	Control
After 18 hours	+	Haze	—	—	+++
After 42 hours	+	Haze	Haze	Haze	++++
After 78 hours	++	++	+	+	++++
After 102 hours	++	++	++	+	++++
After 6 days	+++	+++	+++	++	++++
After 12 days	Trans.	Trans.	Trans.	Trans.	++++

TABLE 3

INHIBITION OF OTHER STRAINS OF PYOCYANEUS BY THE MULFORD FED FILTRATE FROM THE L STRAIN

Filtrate	Parke-Davis		Wisconsin		Buffalo		Mulford	
	1 Drop	Control	1 Drop	Control	1 Drop	Control	0.001 Drop	Control
After 12 hours	++	++++	—	+++	+++	+++	—	+++
After 24 hours	+++	++++	+	++++	++++	++++	+	++++

their controls, were inoculated with 1 loop of 1 loop of a 12-hour broth culture of the respective organism in 8 c c. of broth.

The results of this test shown in table 2 indicate a temporary inhibition of Mulford growth when the filtrate stood at a dilution of 200,000. In one case inhibition was observed in a dilution of 2,000,000. All the tubes which received filtrate presented at the end of 12 days a translucent appearance quite different from that of the control tube.

In the test shown in table 3, the 8 c c. tubes were inoculated with 1 loop of 1 loop of the respective culture. One tube of each pair received 1 drop of the filtrate, except in the case of Mulford, in which the tube received 0.001 drop. From the results it appears that the Parke-Davis strain was inhibited slightly, a Wisconsin strain definitely, a Buffalo strain not at all, and the Mulford strain as before (table 2).

In other tests, which it is unnecessary to report in detail, it was not found possible to raise the L strain filtrate, adapted to the Mulford culture, to a titer much above 200,000 for Mulford. Since, however, it appeared (table 3) that the L filtrate also inhibited the Wisconsin strain, an attempt was made to increase the potency of the L filtrate by feeding with the Wisconsin strain.

L Filtrate Enhanced by Wisconsin Feeding: A tube of the 12th serial L filtrate which was adapted to Mulford was diluted with equal

TABLE 4

INHIBITION TITER OF THE L FILTRATE ADAPTED BY FEEDING TO THE WISCONSIN STRAIN AGAINST WISCONSIN CULTURE

Time of Observation	Dilution of the Lytic Filtrate					
	2,000,000	200,000	20,000	2,000	200	Contro
After 18 hours	—	—	—	—	—	+++
After 42 hours	Haze	Haze	Haze	—	—	++++
After 54 hours	++	++	++	—	—	++++
After 90 hours	+++	+++	+++	+++	+++	++++

parts of broth and fed twice on 2 successive days with about 6 c c. of 12-hour Wisconsin broth culture. The culture was then filtered and fed again on successive days. This process was carried out through 5 filtrations, when the filtrate was tested for its inhibitive power against Wisconsin, with the results shown in table 4.

From the results presented in table 4, it appears that a 2,000,000 dilution of the adapted lytic filtrate inhibited growth when the tubes were inoculated with 1 loop of 1 loop of 12-hour Wisconsin broth. This filtrate was carried on for 12 series of filtrations, but the inhibition titer was not appreciably raised beyond that reported in table 4. This Wisconsin filtrate did not at any time inhibit the L strain, and failed to inhibit the Mulford strain any better than the 22nd Mulford filtrate.

Filtrates from the R Strain: The question naturally arose whether filtrates from the nonlytic strain would show inhibition or lysis of pyocyaneus cultures. The R strain was streaked on agar slants to confirm absence of lytic areas, and from one of these 40 c c. of broth

were inoculated. After 3 days this was filtered, and the filtrate was divided between 3 tubes. The first was inoculated with 1 loop of Parke-Davis, the second with 1 loop of the Mulford, and the third with 1 loop of the R strain. Only the Mulford tube showed inhibition. This tube was filtered, an equal amount of broth added and fed twice with 6 c c. of young Mulford culture. Another filtration followed, and with this filtrate the titration shown in table 5 was obtained.

The R filtrate was not continued in series, the results of the foregoing test being sufficient to indicate that filtrates from the resistant or nonlytic strain, as well as from the lytic strain, possess inhibitive power against a sensitive culture. In other tests the R strain was not inhibited by its own filtrate, as one would expect. When the R filtrate was fed with Wisconsin culture, a filtrate more active against this strain was obtained, as was the case with the L filtrate.

Filtrates from the Mulford and the Wisconsin Strains: If a lytic filtrate could be obtained from a culture such as the R strain, which

TABLE 5

INHIBITION FOR MULFORD CULTURE OF THE MULFORD FED R FILTRATE

Time of Observation	Dilution of the R Filtrate		
	1:200	1:66	Control
After 12 hours	++	+	++++
After 24 hours	+++	++	++++

showed no sign of lytic action in broth or on agar slants, it seemed possible that the apparently normal strains, Mulford and Wisconsin, which were inhibited by the L filtrates and the R filtrates, might themselves be contributing to the inhibitive action through the elaboration of lytic substances. To control this point, filtrates were produced from both of these strains and were alternately fed and filtered through 3 series. At the end of this time neither filtrate possessed any inhibitive action on either the Mulford or the Wisconsin strain. The same was true with a filtrate from the Parke-Davis culture, which was slightly sensitive to the L and the R filtrates. Moreover, it was found impossible to adapt an L filtrate already partially adapted to the Mulford to the Parke-Davis strain as a result of a series of 8 passages. The strain remained resistant.

Summing up the results of the study of the influence of culture filtrates, it may be said that filtrates from both the L and the R components of the original culture of pyocyaneus were found to possess

marked inhibitive power for sensitive strains such as the Mulford and the Wisconsin, but not for the homologous culture, and not for other resistant cultures, such as Parke-Davis and Buffalo. It may be added here that in all cases the development of a lytic filtrate of high titer takes place slowly. Methods which can be employed with immediate success when dealing with the d'Herelle anti-Shiga lytic principle, serve in the case of pyocyaneus not to increase the activity of the filtrate, but to decrease it. In raising its titer, one must proceed with caution in order not to transcend by dilution the effective limits of action of a principle which increases slowly in amount and energy.

THE PRODUCTION OF LYTIC AREAS THROUGH THE AGENCY OF LYTIC FILTRATES ACTING ON SENSITIVE CULTURE

When a subculture was made from a lytic area of the L strain on agar, the new culture was likely to present no more lytic areas than the original, and it might present fewer. When a subculture was made from apparently normal growth in the space between lytic areas, the new culture might present either more or fewer lytic areas than the original; and it was likely to present just as many lytic areas as the subculture made from a lytic area. From these results one could draw one of two conclusions: (1) Either the lytic areas did not represent the point of action of individual lytic agents, or (2) the number of lytic agents in the culture mass found no adequate expression in the number of visible lytic areas, at least when occurring in association with the L strain. The following tests were made to verify one or the other of these assumptions:

Influence of L Filtrate on the Lytic and the Nonlytic Strains.—Two broth tubes were inoculated respectively with the L strain and the R strain, and incubated over night. To each was added 1 drop of the L filtrate fed with the L strain and active against Mulford, but not against the L strain itself. After the lapse of 15 minutes a loop from each tube was streaked over the surface of an agar slant, which was then incubated over night. The slant culture from the L strain broth plus the drop of L filtrate showed about the normal number of large lytic pockets; they were not increased by the additional lytic agent. The slant culture from the R strain broth plus the drop of L filtrate showed at first a slightly modified growth, the surface having a dull luster, but eventually it became normal and showed no lytic pockets. Manifestly, then, the addition of more lytic agent to lytic culture failed to make it more lytic; and similar addition to a nonlytic culture failed to cause

the manifestation of lytic areas. In these cases, therefore, the presence of lytic pockets in a certain number could not be accepted as a criterion of the "number of lytic agents" present in the culture.

Influence of Lytic Filtrates on Sensitive Strains.—It is conceivable, however, that a fairer test would involve the addition of L filtrate to a sensitive culture. A test of this kind was made, employing the L filtrate adapted to (1) Mulford and (2) Wisconsin. In the first case the inhibition titer of the filtrate was about 200,000; in the second about 2,000,000.

Enumeration Method: The broth tubes were inoculated with the Mulford and Wisconsin strain, respectively, and incubated for 12 hours. Then into the Mulford tube was placed 1 loop of the L filtrate built up on Mulford, and into the Wisconsin tube 1 loop of the L filtrate built up on Wisconsin. From each tube 2 loops of culture were streaked over an agar slant which was incubated. After 12 hours both tubes showed good growth, but the surface was studded with from 60 to 80 lytic areas varying in size from the lowest visible limit to 2 mm. In these areas no culture was observed, but the bronze sheen seen in the lytic pockets of the L strain was absent. After 3 or 4 days these areas became more or less overgrown by surrounding culture.

This test was repeated many times with variations. The lytic areas in the Wisconsin culture were larger and more clear cut than in the Mulford. The number was always directly proportional, either to the amount of the L filtrate added to the broth culture before streaking, or to the amount of inoculated culture streaked. The number of lytic areas was not modified by altering the concentration of bacteria to which the loop of L filtrate was added in the pyocyaneus culture. These results are in harmony with those of d'Herelle embodying the action of his lytic principle on cultures of the Shiga dysentery baccillus.

Lytic Areas Produced on Lytic Agar: A similar production of discrete lytic areas in the sensitive strains, Mulford and Wisconsin, was obtained in another way. To a tube of 10 c c. of melted agar, cooled to 50 C., was added one drop of the L filtrate built up against Wisconsin. The tube was rotated to accomplish a thorough mixing and then slanted. When cool, 2 loops of a broth culture of Wisconsin were streaked evenly over the surface and the culture incubated over night. In the morning there were present from 50 to 100 lytic areas against the background of culture. In patches they had become confluent and destroyed the growth over wide areas. When the tubes were left to themselves, the nonlysed growth from the borders of the tube,

or from nonlysed islands of culture, grew slowly over the lysed area. If the original agar tube of Wisconsin culture was inoculated with more than 1 drop of L filtrate, more lytic areas resulted; if it was inoculated with less than 1 drop, fewer lytic areas developed. The number of areas in this case was always proportionate to the amount of L filtrate employed.

Characteristics of the Lytic Areas: When these lytic areas are examined with a hand lens, they are found to differ markedly in appearance and behavior from the corresponding areas produced by d'Herelle's lytic filtrate on Shiga cultures. In the latter case, the lytic colonies are always round, and they never become overgrown by culture from their periphery, although secondary cultures may spring up from their surface. The lytic areas in the pyocyaneus cultures are seldom round when they are young; they may be bar-shaped, star-shaped, spider-shaped or quite irregular in outline. They vary from microscopic size to 3 mm. As they grow older, there is a tendency to become round. Sometimes when they are young, more commonly when they are older, they appear to be surrounded by a gray band or halo which follows closely the outline of the colony. This band gradually encroaches on the lytic surface and represents the process of invasion of the lytic area by outside culture. Eventually, the area becomes gray or hazy and may finally almost disappear by merging into the surrounding culture mass.

Effect of Lytic Action on Sensitive Strains.—When subcultivations from agar slants carrying pocketed cultures of the Wisconsin strain are made to fresh agar slants, a still different type of growth appears. The first generation on agar presents a growth which can best be described by the term "mealy." The normal glistening luster and the smooth, homogeneous growth are replaced by a dull luster and a granular surface appearance, with irregularities in the thickness of the growth. If subcultures of this tube, in turn, are made to slant agar, the third generation presents still another appearance: the growth is smoother and more homogeneous, with a less dull luster, and the surface is marked by a variable number of minute depressions or "pits," which may disappear with the further growth of the culture. If there are many such pits, they occur over the entire surface; if they are few, they appear only at the middle and bottom of the slant; if very few, they occur at the bottom only. When subcultures of this tube, in turn, are made, the fourth generation growth may present no evidences of lytic action, or there may be a few lytic pits at the base of the tube. The consistency and surface are otherwise normal, but the growth

develops only the yellow pigment. Thus it appears that, while the lytic characteristics of the original L strain could be perpetuated on slant agar for many generations, the culture in the meantime retaining its power of forming an intense blue-green pigment, the lytic characteristics which were "impressed" on the Wisconsin strain were lost within the range of 4 or 5 generations.

When such pocketed cultures of Mulford or Wisconsin strain are plated, or when plates are poured from broth tubes of Mulford or Wisconsin strain to which L filtrate has been added, the results are quite different from plates poured from the original L culture, which, as will be recalled, gave both pocketed and nonpocketed colonies. In the case of Mulford or Wisconsin, no pocketed or "infected" colonies, nor any colonies giving evidence of particulate or mass lysis, appear. All colonies are apparently the same. They are not, however, the same as the colonies of the original Mulford or Wisconsin culture, for they yield no blue-green pigment, only the yellow-green; and they are more resistant to the lytic action of the L filtrate built up on the Wisconsin and on the Mulford strains. They have been changed into a nonlysogenic R type.

When a subcultivation to broth is made from an agar slant carrying the first pocketed culture of Wisconsin strain, and subsequent broth cultivations are made in series, the lytic agents are perpetuated for several generations and will appear on agar slants. The broth tubes themselves, however, show neither lysis nor inhibition of growth. In them the blue-green pigment is never formed.

One concluding observation on the varying evidences of the presence of a lytic agent in agar cultures of pyocyaneus not showing frank lysis may be mentioned: A normal pyocyaneus culture possesses a glistening luster. If this luster disappears, or is replaced by a dull or granular surface, or a mealy, moth-eaten or wrinkled growth, one may be certain that a lytic agent is operating in the bacterial mass. On the contrary, a glistening luster is no evidence that a lytic agent is absent, and this is particularly true when the blue-green is suppressed in favor of the yellow-green pigmentation.

Briefly to summarize this section: It is shown that there is no fundamental difference between the mode of action of the pyocyaneus lytic filtrates and the lytic principle of d'Herelle when the former is permitted to operate on a favorable, sensitive strain of pyocyaneus. The differences that do exist can easily be explained on the basis of differences in energy and in speed of the lytic action. These are doubt-

less determined in part by variations in the "virulence" of the lytic agent and in part by variations in the reaction of the cultures to the lytic stimulus.

INFLUENCE OF THE L FILTRATE ON OTHER BACTERIAL SPECIES

Several investigators, notably Emmerich and Löw, have reported the antagonistic influence of pyocyaneus filtrates on the growth of B. anthracis and other organisms. These authors found that when old cultures of pyocyaneus which had come to possess a gelatin-like consistency were evaporated down to one-tenth their original volume, this material possessed bactericidal powers; and from it they were able to obtain, by precipitation, an enzyme which they called pyocyanase, and which possessed marked lytic power for anthrax bacilli and some other bacteria.

In view of the present recognition of a lytic phenomenon occurring in pyocyaneus cultures, and also the recognition of the fact that the anthrax bacillus is susceptible to transmissible autolysis by the bacteriophage, the question arises as to the influence of the L filtrate on anthrax and other cultures. Are anthrax bacilli destroyed by the lytic agent present in the filtrate, or by the pyocyanase present in the filtrate, or perhaps by both? Or may the lytic agent be bound up in some way with the pyocyanase or with other proteases of the cell? This problem is somewhat difficult to approach because, although there can easily be demonstrated in the L filtrate both a lytic action for pyocyaneus and a protease having the power of digesting gelatin without the aid of living bacteria, the separation of these factors, so that they can be made to act separately has encountered obstacles not yet overcome.

Attempt to Distinguish the Lytic Agent from the Protease.—At the outset the idea suggested itself that if it could be shown that the lytic agent and the protease or pyocyanase (which have never been shown to be distinct entities) possessed different thermal death points, or at least that the protease was destroyed at a lower temperature than the lytic agent, the independent action of these might be studied more satisfactorily. Accordingly, a few preliminary tests were made in which samples of the anti-Mulford L filtrate XIV were heated at temperatures between 65 and 90 C. for 30 minutes, then tested simultaneously for their inhibitive action on a sensitive strain and for their proteolytic

action on gelatin. The detailed results of this phase of the study will be reserved for later publication, but may be mentioned briefly at this time.

A lytic filtrate which inhibits temporarily the growth of a sensitive strain of pyocyaneus in a dilution of 2,000,000, and which slowly digests gelatin when a few drops are placed on the top of a gelatin column, loses its power to do both when heated for 30 minutes at 76 C. Lower temperatures permit the partial survival of both characteristics. At 74 C. the ability is not destroyed, but greatly weakened.

The results of several tests thus indicate that the proteolytic agent which digests gelatin and may be related to the "pyocyanase" of Emmerich and Löw,[5] and the agent which inhibits the growth of pyocyaneus and causes lytic pockets in sensitive cultures, possess approximately the same thermal death point. When the power to accomplish one is lost, the power to accomplish the other also disappears. Notwithstanding these results, it was decided to test the action of the L filtrate with whatever active factor for lysis it might contain against cultures of B. anthracis.

Action of the L Filtrate on B. Anthracis.—In one of 2 tubes of broth is placed 1 c c. of the L filtrate against pyocyaneus. Both tubes are then inoculated with 1 loop of a young culture of B. anthracis. For some time subsequently both tubes appear to grow equally well. After 3 weeks at room temperature, however, the tube which received the L filtrate is seen to have much less growth sediment than the other tube. Both tubes are filtered, and fresh broth is added at the rate of about 3 : 1 of filtrate. The pair of tubes are again inoculated with 1 loop of fresh anthrax culture, incubated for 24 hours, and then left at room temperature. After a week's time the same difference is noticed between the tubes, but the difference is greater: the control tube contains visibly heavier growth than the L filtrate tube. This process is repeated, using larger amounts of inoculum, but with similar results. Finally, to the last filtrate were added 5 drops of a heavy washed-up agar slant suspension of anthrax; no growth resulted.

Action of the L Filtrate on Other Organisms.—Similar tests were applied to B. prodigiosus, B. ruber, B. rubidus, Sar. lutea, Sar. aurantiaca, B. coli, B. typhosus, B. dysenteriae (Shiga) and B. fluorescens. Only in the last instance was any growth-modifying influence observed from the use of the L filtrate in a single test. In case of fluorescens, a

[5] Ztschr. f. Hyg. u. Infektionskr., 1899, 31, p. 1.

filtrate which would inhibit growth of a broth culture of the organism was not obtained, but evidence of lysis was gained in another way. A broth culture was grown for 12 hours, when a subculture to slant agar was made. Directly afterward, to the broth tube were added 3 drops of the L filtrate, mixed, and the tube incubated at 37 C. for 20 minutes. Then 2 loops of the mixture were streaked on an agar slant, and this, together with the first agar slant, was incubated for 12 hours. When examined at the end of this time the control tube (1) gave a medium, homogeneous growth, while the slant made from the mixture (2) showed a multitude of minute pits over the surface. These disappeared within the next 12 hours, and thenceforward both tubes presented the same appearance.

It may be added here that the following lytic filtrates from various sources were tested against sensitive strains of pyocyaneus without calling forth any sign of lytic action:

(1) A d'Herelle anti-Shiga filtrate (original from d'Herelle's laboratory).
(2) An antityphoid lytic filtrate obtained from a case diagnosed as colitis.
(3) An antityphoid lytic filtrate from a case diagnosed as pyelonephritis.
(4) An anticolon lytic filtrate from a case diagnosed as pyelitis.
(5) An anti-Shiga lytic filtrate from a case diagnosed as amebic dysentery.
(6) An anticolon-dysentery lytic filtrate from a septic tank.

In the case of the d'Herelle lytic agent the filtrates were carried through 6 series of filtrations, but eventually gave no evidence of lysis or even of inhibition.

THE RELATION BETWEEN THE LYTIC AGENT, PYOCYANASE AND THE PROTEASE IN PYOCYANEUS FILTRATES

It is well known that sterile pyocyaneus filtrates usually have the power of digesting gelatin. This was proved to be the case with a filtrate of the L strain adapted to the Mulford strain. It was also known that this filtrate possessed inhibition power over the Mulford and Wisconsin strains in fairly high dilutions. The question arose as to whether the experience of digesting gelatin, through the presumable action of the protease of the filtrate, would influence in any way the power of the filtrate to inhibit the growth of the Mulford culture.

Effect of Gelatin Liquefaction on Activity of the Lytic Agent.—To the top of a 5 cm. column of gelatin was added 0.25 c c. of anti-Mulford lytic filtrate of the fourteenth series. Liquefaction was observable within 48 hours and proceeded slowly, giving a linear liquefaction of 4.5

cm. in 64 days. When the liquefaction had reached 1 c c. (volumetric), the inhibitive power of the liquefied gelatin was tested against the Mulford strain. Three tubes were set up as follows:

(1) 8 c c. of broth containing sufficient L filtrate XIV to give a dilution of 1 : 1600.

(2) 8 c c. of broth containing 0.1 c c. of the 1 c c. of liquefied gelatin (making a hypothetical dilution of the original filtrate of 1 : 320).

(3) 8 c c. of broth without addition of either gelatin or filtrate (control).

To each tube was added 1 loop of 1 loop of young Mulford culture in 8 c c. of broth, and the tubes were incubated at 37 C. At the end of 24 hours (1) showed a slight haziness, (2) grade 4 clouding, and (3) grade 4 clouding. Apparently some agency had caused the suppression of the inhibitive action of the lytic agent in the liquefied gelatin, so that it was no longer active even in a 1 : 320 dilution.

Since, however, in the case of pyocyaneus, as has been shown previously, the failure of inhibition of broth cultures may not be a fair criterion of the absence of lytic agent, a test was performed to ascertain the presence or absence of lytic areas in agar slants inoculated with the liquefied gelatin plus normal sensitive culture. One drop of the liquefied gelatin was placed in a tube of broth containing a 16-hour growth of Wisconsin strain. From this tube 2 loops were spread upon slant agar and incubated. No sign of lytic action appeared, thus indicating that the lytic agent had disappeared from the tube of liquefied gelatin.

Since such a result might be due to the action of gelatin itself, without regard to the action of the protease or other substances present in the lytic filtrates, a test was made of the inhibitive action of plain gelatin filtrate mixtures employed in the inhibition tests before the protease had time to act. Accordingly 6 tubes, each containing 4 c c. of broth, were set up with the following additions:

(1) 1/25 c c. of a mixture of 1 part filtrate to 19 parts melted gelatin; final dilution of filtrate = 1 : 1600.

(2) 1/25 c c. of a mixture of 1 part filtrate to 9 parts melted gelatin; final dilution of filtrate = 1 : 800.

(3) 1/25 c c. of a mixture of 1 part filtrate to 3 parts melted gelatin; final dilution of filtrate = 1 : 266.

(4) 1/25 c c. of a mixture of 1 part filtrate to 1 part melted gelatin; final dilution of filtrate = 1 : 80.

(5) 2/25 c c. of a mixture of 1 part filtrate to 1 part melted gelatin; final dilution of filtrate = 1 : 40.

(6) Control tube; no filtrate or gelatin added.

Each tube was inoculated with 1 loop of 1 loop of 12-hour Mulford culture in 6 c c. of broth, and the tubes were incubated at 37 C. At the end of 20 hours the results were as follows: (1) clouding, grade +; (2) clouding, grade +; (3) marked haziness; (4) slight haziness; (5) slight haziness; (6) clouding, grade + + +.

From these results it is clear that gelatin by itself, in the brief time of contact allowed in this test, did not interfere appreciably with the action of the lytic filtrate in its inhibition of Mulford growth. The point involving the loss of inhibitive action of the lytic filtrate in the liquefied gelatin is still under investigation.

DISCUSSION OF RESULTS

Earlier Observations on Pyocyaneus.—Pyocyaneus has long been recognized as a strongly proteolytic micro-organism whose action is due to an active protease. Casein, serum, and gelatin, and sometimes fibrin, are digested. Emmerich and Löw (1899)[5] isolated from old cultures an enzyme which had the power of digesting bacteria and was called by them pyocyanase. The relation of this enzyme to the protease is not well established. Bullock and Hunter (1900)[6] observed in cultures a hemolytic substance which they termed pyocyanolysin; Jordan, however, believed that the hemolytic action was due to the intense alkalinity of old cultures. Gheorghewski (1899)[7] observed in cultures a substance which destroyed leukocytes. A soluble toxin was studied by Wassermann. Of the pigments formed by pyocyaneus, fluorescin and pyocyanin have been studied by many investigators. None of the earlier investigators have, however, so far as I am aware, commented on the peculiar evidences of autolytic action now described, although it would seem that it must have been observed many times without a recognition of its actual significance. Indeed, Jordan, in 1899,[3] refers to a culture, "B. pyocyaneus, Rush," obtained a year previously from the body of a guinea-pig (that had died after inoculation with a fragment of diphtheric membrane) which suggests the L type. For instance, "Rush" was the only culture which formed a "thin, delicate film which rapidly covers the whole surface (agar slant) and has a pronounced metallic lustre." "Rush," moreover, was the first to produce color and the only culture to form a deep blue-green pigment. It was also the only culture to give the chameleon phenomenon of Ernst.[2] It vigorously reduced nitrates, liquefied gelatin, and formed indol to a greater extent

[6] Centralbl. f. Bakteriol., I. O., 1900, 28, p. 86.
[7] Ann. de l'Inst. Pasteur, 1899, 13, p. 298.

than any other strain. Jordan's notation of the rapid expansive growth of this culture on slant agar is particularly significant, as will be shown later. It is probably that this culture was under the influence of a lytic stimulus.

It will readily appear from the foregoing presentation that the phenomenon now under discussion shows resemblances, not only to the lytic phenomenon of d'Herelle,[1] but even more to the phenomenon described some years earlier by Twort [8] (1915). The latter, it may be recalled, attempted to cultivate glycerinated calf vaccine on agar. In some of the cultures micrococci grew, and these colonies became glassy or transparent, this appearance beginning at the edge and extending over the entire colony. If the transparent material was diluted and filtered and a drop added to an agar slant, growth of a similar organism was prevented. The transparent material passed Chamberland F and B filters, and one drop of the filtrate spread over an agar slant made the medium unsuitable for the growth of the micrococcus. The culture would start to grow, but would soon be covered with transparent points, which would rapidly extend over the whole growth. The number of "points" depended on the dilution of the filtrate. This colony infection could be transplanted, but the transparent material itself would not grow on any medium. If small areas of culture happened to be left in an "infected" slant agar tube, these areas would start to grow again and extend over the transparent portions of the culture. The transparent material maintained its vitality for at least 6 months. It was destroyed by heating at 60 C. for one hour.

D'Herelle [9] (1922) has maintained that the observations of Twort are not concerned with the action of the bacteriophage. Others (Gratia,[9] 1922), however, believe that the Twort phenomenon and the d'Herelle phenomenon are identical. It will be of interest subsequently to compare the outstanding features of the lytic action in pyocyaneus cultures with the case described by Twort and also with the action of d'Herelle's Bacteriophagum.

More recent literature, appearing while the present study was under way, afforded precedent for regarding the lysis of pyocyaneus as a phenomenon involving the activity of a lytic agent comparable with d'Herelle's Bacteriophagum. Thus, Blanc [10] (1923) published a brief account of modified cultures of pyocyaneus. While the normal colonies

[8] Lancet, 1915, 189, p. 1241.
[9] Brit. Med. Jour., 1922, p. 289.
[10] Compt. rend. Soc. de biol., 1923, 88, p. 52.

were of large diameter, homogeneous and brightly pigmented, the "modified" colonies were small, dull and folded. Seeding from these gave a mixture of normal and modified culture. The "modified" cultures showed cuplike depressions, representing thinnings of the microbial layer. On the surface of these areas the pyocyanin and the fluorescent pigment were more or less abundant. Under the action of filtrates the culture was further modified to give a growth in which both pigments were suppressed, a characteristic which remained stable for at least 15 passages. Along with their loss of pigment, the modified cultures also showed loss of their power of liquefying gelatin.

Somewhat later Quiroga[11] (1923), while examining cultures from pus of a case of osteomyelitis, found, among other organisms, pyocyaneus colonies. From this developed two strains: one resistant, producing only a yellow-green pigment; the other lysable, producing the blue-green pigment. Lytic filtrates were obtained which caused the lysis of the sensitive strain in from 16 to 20 hours. The nonlysable strain was more virulent for rabbits than the lysable. Also Cancik[12] (1923) has briefly reported observing colonies of a culture of pyocyaneus containing silver flecks from which lytic areas develop.

Still later Hauduroy and Peyre[13] (1923) presented data on the lysis of pyocyaneus cultures obtained (1) from a case of osteomyelitis of the tibia and (2) from epithelioma (ulcus rodens) of the temporal region. The agar cultures gave two kinds of colonies. One was "normal," the other contained lytic areas showing a metallic sheen. Filtrates from the lytic strain caused the lysis of several pyocyaneus cultures. The lytic agent was inactivated by a temperature of 80 C., but was again active after three passages in broth with intermediate filtrations.

It seems probable that the cases mentioned above represent the same phenomenon reported in this paper, and that all are examples of an autolytic process depending on the same causative factors present in the phenomena reported by Twort and by d'Herelle. The analogy with the Twort phenomenon in particular is too obvious to require further exposition at this time; the outsanding feature of coincidence is that of particulate lysis and of the "transparencies," or the so-called "appearances" of some writers. The analogies with the d'Herelle phenomenon are less obvious and require examination in greater detail.

[11] Ibid., p. 363.
[12] Jour. Am. Med. Assn., 1923, 80, p. 970 (abstract).
[13] Compt. rend. Soc. de biol., 1923, 88, p. 688.

Comparison of the Outstanding Features of Pyocyaneus Autolysis with the d'Herelle Phenomenon.—If we accept the outstanding features of transmissible bacterial autolysis in the d'Herelle sense as comprising (1) inhibition of growth by sterile filtrates, (2) lysis in series, and (3) occurrence of particulate lytic areas, we may now consider the lysis of pyocyaneus in relation to these main points.

When pyocyaneus lytic filtrate is added to a tube of broth, and this tube is subsequently inoculated with a sensitive culture of pyocyaneus, growth does not appear for several days. If to the 8 c c. broth tube, a moderately large amount of filtrate is added, say a 0.1 c c., the growth may be inhibited for 4 or 5 days. If to the broth tube a smaller amount of filtrate is added, say 0.01 c c., the growth may be inhibited for 3 days. If, however, to the broth tube 0.25 c c. or 0.5 c c. is added, or even 50% by volume of the lytic filtrate, no greater inhibition is gained; the growth still appears in 5 days if the seeding is light (1 loop of 1 loop into 8 c c. broth tube). In other words, no pyocyaneus lytic filtrate has been found which had the power to kill all the organisms in the tube; growth always occurs in some measure, and a final, partial clearing occurs only when fairly large amounts of lytic filtrate are employed, and after considerable time.

These results differ from the d'Herelle phenomenon in several respects, but the differences appear to be of a quantitative rather than a qualitative order. Absolute inhibition against Shiga culture is easily attainable, and it may be accomplished by the use of very high dilutions of a lytic filtrate. This has not been attained with pyocyaneus filtrates whose extreme action in inhibition has been placed at about 1 : 2,000,000. In the case of pyocyaneus, moreover, absolute inhibition was found to be unattainable with the cultures used, regardless what concentration of the lytic filtrate was employed.

If a small amount of active anti-Shiga bacteriophage, say 0.01 c c. of a 10^{-9} dilution is placed in a broth culture of the organism, the organisms will eventually break up, and the tube will become clear. If 0.01 c c. of 10^{-6} is added, the process is more rapid; and if 1 c c. of 10^{-3} is added, lysis takes place within a few hours. The age of the culture used for such tests may be from a few hours to 2 days. If, however, any similar amounts of the pyocyaneus lytic filtrate are added to a young broth culture, lysis does not occur, although lysis may occur when relatively large amounts of filtrate are added. Even here, however, lysis takes place only after days or weeks, leaving the medium a clear iridescent green. Typical pyocyaneus lysis therefore was found to

occur, but only under limited and most favorable conditions. It is not by the simple lytic test that the presence of a lytic agent for pyocyaneus can be recognized, or its relative potency established.

In a comparison of the present case with the d'Herelle phenomenon, it is the production of the lytic areas by the employment of the L filtrates on sensitive cultures of pyocyaneus that really brings the two into unity. Between the methods and the main results there is no essential difference; and the minor differences can be explained. Although the behavior of the lytic culture in the perpetuation of the lytic areas is different from anything observed in the lysis of the dysentery organism, the action of the pyocyaneus lytic agent in filtrates supports the view that the lytic areas have their origin in single "lytic centers" just as truly as in the case of the lytic areas in a dysentery culture under the action of the d'Herelle bacteriophage.

In a comparison between the pyocyaneus lytic areas with those produced by a d'Herelle filtrate (anti-Shiga, for instance), one striking difference obtains; and it may be said that the same difference was observed by Twort in the lytic areas in his culture of staphylococcus. It concerns the point of the secondary overgrowth of lysed areas. D'Herelle states that lysed areas were never overgrown from the surrounding culture. Twort states that if small areas of culture happened to be left in a slant agar tube, these areas would start to grow again and extend over the transparent portions of the culture. In the lysis of pyocyaneus, a secondary growth often appears over the area of lysis. This new growth is derived from two sources: (1) the edge of the lytic area, from which the "normal" culture may gradually encroach on the lytic site; (2) the floor of the lytic site, on which a new growth may spring up, raising, as it increases in thickness, the fluorescent film, which covers the area. By such a process, the fluorescent film, which at the stage of maximum lysis lay on the floor of the lytic pocket, comes to lie again on the same level with the surrounding "normal" culture. This phenomenon differs only in degree from that which I observed repeatedly in the case of lytic areas in a Shiga culture, caused by the action of d'Herelle's filtrate. On such areas, after many days, colonies appear. These are of the "modified type" and never over-run the lytic area, but remain for the most part discrete. In the case of the Shiga lysis I have never observed encroachment from the border of the lytic colony. The difference between Shiga and pyocyaneus in this point seems to be explained by difference in the energy of the lytic agents, or by difference in the resistance of the

respective cultures, or by both. It is not of sufficient significance to be regarded as evidence that these two manifestations of lysis are not attributable to the same kind of causal agency.

We may now attempt to ascertain how the explanation by d'Herelle of the mode of action of the bacteriophage as studied by him may be modified to explain several of the peculiarities of pyocyaneus lysis. We may consider first the matter of the so-called lytic or pocketed colonies of pyocyaneus, comparable in many ways with Twort's "infected" colonies of cocci. Nothing exactly analogous to these in the case of the intestinal organisms susceptible to the action of the d'Herelle bacteriophage has been described. And we shall see that, although the explanation of all the peculiarities of pyocyaneus lysis introduces complexities not found in simple bacterial autolysis as outlined by d'Herelle, there are no far-reaching differences between the Twort phenomenon, the d'Herelle phenomenon and the present case.

Tentative Explanation of Pyocyaneus Lysis.—Let us review again the facts before us. From the lytic strain of pyocyaneus a plate is poured. An organism becomes isolated on that plate and produces a colony which increases in size. At some point in the development of that colony a lytic spot appears, perhaps in the center, perhaps at the margin, perhaps in some intermediate position of the colony. The colony undergoes rapid development. The lytic area expands into a pocket, develops to its limit, and then, for a time at least, remains in equilibrium with the surrounding culture. At other points in the colony other lytic centers arise. A conflict appears to ensue between the lytic agent and the bacteria. Eventually either the colony or the lytic areas gain the ascendency, and the colony as a unit thing either survives or perishes. This colony-infection might be regarded as the epitome of an infective process in a living animal; and from such a conception, involving postulates of the immunity of bacteria, and the like, one could easily enter a field of alluring but perhaps not particularly helpful speculation. Several questions of serious import, however, present themselves. Whence came these lytic pockets, these progressive erosions? We certainly must trace them back to the original organism from which the colony developed. That living cell must have harbored the potential possibility of developing particulate areas of colony lysis. In what form did this potential possibility exist? How was it transmitted through millimeter after millimeter, or even centimeters, of homogeneous culture, through generation after generation of growing cells,

without revealing itself? And, finally, what enabled it to come into expression, sometimes with almost explosive suddenness, far from the place where the original bacterial cell came to rest on the agar surface?

Any hypothesis purporting to explain the nature of the lytic action of pyocyaneus, or of transmissible lysis in general, must supply answers to these questions. Although the rather superficial nature of this first examination of the chief facts of pyocyaneus lysis scarcely warrants an attempt to formulate an explanation of the phenomenon, much less an explanation of transmissible lysis in general, a critical examination of the facts presented may serve to throw some light on one or two points that at present are under discussion by other workers.

The first point that invites attention is the following: When a lytic agent is incorporated within an organism of the L type, the lytic agent may be carried by the cell and by its descendants for a considerable time without causing any observable manifestation of lysis. In evidence of this one may note that, in pyocyaneus colonies arising presumably from a single organism, macroscopic lysis often does not start at the center of the colony, but near one edge. It may start at points millimeters away from the center, and after the interposition of numerous generations of cells. To these areas where lysis first shows itself, the lytic agent must have been transported through chains of growing cells, descendants of the first cell. Not only is the lytic agent carried forward by the advance guard of cells, but it remains also along the line of march, incorporated in all those cells that extend back to the starting point at the center of the colony; it is a part of the cell organization. Although these cells do not show lysis, the lytic agent can always be recovered from them as readily as from the cells in locations where marked lysis may be in evidence. Moreover, these cells may be precipitated into lysis merely by transferring them to a fresh medium of suitable reaction.

What is the relation of the lytic agent to the organism during its obscure passage from cell to cell through hundreds of generations? From the orthodox point of view it is conceivable that two things might be happening: (1) an increase in the amount of lytic agent within the cells, (2) an increase in its "virulence" for the cells. As a result of one factor or the other, or of both factors, the time comes when the cell is destroyed and the destruction extends to other cells in the immediate neighborhood, with the result that a lytic pocket or zone is produced. These conceptions obviously give no more favoritism to the view that

the lytic agent is a living virus than to the view that the lysis of the cell is the result of an intensified autolytic action, a stimulated self-destruction. Either would explain the case.

D'Herelle, in working with his antidysentery bacteriophage has recognized that an appreciable time is required for the lytic principle to become incorporated with the bacterial cell and evoke lysis. This would seem to correspond with the so-called "latent period" of enzyme action, although d'Herelle assumed it to be concerned with the multiplication of the lytic agents within the bacterial cell. After a certain time they were assumed to have attained such numbers that the cell dissolution took place. In d'Herelle's case, therefore, it would seem improbable that a dysentery bacillus would have time to divide after the lytic agent had been incorporated within it. Destruction of Shiga cultures therefore proceeds at a rapid rate; there would appear to be no carrying along of the lytic agent through generations of cells, except perhaps in a resistant strain. The lysis of dysentery cultures is notable for the speed of the reaction.

But it is not inconceivable that, in the general variation of things, there could exist such a thing as a "low-speed" lytic process, whose full effects obtain only after a slow building-up to the threshold of action; this condition might exist if we were dealing with an autolytic enzyme. During the building-up process the presence of an increasing amount of the lytic agent within a bacterial cell might in no way hinder normal growth; it might even stimulate or be accompanied by, growth far beyond the normal rate. And, in this connection, it has been reported in these pages that no culture of pyocyaneus studied possessed such growth-energy as that observed in the lytic strains, where artificial colonies that were undergoing progressive lysis commonly attained diameters of 60 to 80 mm. The colonies of the R strain, also carrying the lytic agent, seldom grew to more than 18 to 20 mm. We are led to assume, therefore, that slow increase of the lytic agent through generations of cells, serves, first to stimulate growth; or, at least, is accompanied by excess growth; but up to a certain point at least it works no visible harm on these cells; they approach what might be termed the threshold of lysis. Then something causes an "explosion," and a rapid and complete lysis over broad areas manifests itself. This phenomenon contains the essence of the problem. The question is: What serves as the detonator for the primed or sensitized culture?

The foregoing considerations serve to suggest another possibility which may be stated as follows: When the rapid rate of multiplication

or generation of the lytic agent within the bacterial cells from generation to generation has more than compensated for the diluting effect of successive rapid cell divisions, the dissolution of the cell is brought about, either spontaneously or under the influence of a second factor. This is likely to occur at the same time in several cells in the same immediate neighborhood, these cells having together reached what might be termed the "threshold of lysis." The sudden liberation of a fresh lytic agent from these few cells is sufficient to throw the balance, so to speak, in many neighboring cells which are also on or near the threshold of lysis; and these, in turn, effect lysis in others. In this manner particulate lytic corrosions are produced, which may extend their boundaries within wide limits.

With reference to the possible rôle played by autolytic enzymes, it must be regarded as a fact of prime significance that the agent or the condition which represses the lytic manifestation in resistant cultures of pyocyaneus also represses its other enzymatic activities, such as the liquefaction of gelatin and casein and its pigment production. And it must be regarded as a fact of equal significance that when a culture of pyocyaneus is in that physiologic state in which high efficiency in enzyme action in general is present, that culture is also either actively lytic (showing particulate areas of frank lysis) or is highly susceptible to the influence of the lytic agent (sensitive state, on the "threshold of lysis," etc.). Such a correlation of circumstances lends weight to the view that the thing which effects lysis, under proper instigation, is the intensification of one of the normal enzymatic properties of the bacterial cell whose rise and fall in cell life keeps pace with the functioning of other enzymes. What effects one effects all. When autolysis is increased, the pyocyanigenic activity is increased; when one is repressed, the others are repressed. From this point of view the problem of transmissible autolysis appears to be definitely concerned with the balance between the anabolic and katabolic activities of the bacterial cell.

Bearing on this point also are the observations on pyocyaneus which suggest that the final destruction of the bacteria is perhaps not the most significant point in the lytic process. Is it not possible that of greater significance, in any attempt to explain the phenomenon of transmissible lysis, is the antithetical fact that the actual lytic action is first preceded by a vigorous stimulation of the metabolic rate and the promotion of cell growth, rather than of cell destruction? This point was first mentioned by Bordet for B. coli, in contrast to d'Herelle's

view that destruction of bacteria by lysis starts at once. In pyocyaneus lysis, the phenomenon of preliminary growth is particularly in evidence. From this point of view, the primary influence of the lytic agent (which in this case would appear to be catalytic) is to speed up a normal function of the bacterial cell, to heighten the energy of cell life and growth, while the subsequent fact of lysis, though inevitable, is nevertheless secondary. The problem seems to lie in the realm of intracellular digestion; in the relation between the anabolic and katabolic processes of cell life.

In other words, when the lytic process is retarded as in the case of pyocyaneus, so that we can observe all the successive changes, we can perceive that it comprises the following stages:

(1) Origin of the autolytic agent within the bacterial cell in response to some growth stimulating factor in its nutritive environment.

(2) Slow or rapid development or generation of the autolytic agent through successive generations of cells, during which time they present the feature of excess growth and gradually approach the lytic threshold.

(3) The sudden lysis of one or more cells, either spontaneously or under the stimulus of some substance or condition present in the medium, with the consequent sudden liberation of a mass of the lytic agent.

(4) The consequent rapid lysis of many neighboring cells that are also on the threshold of lysis as a result of the outburst of lytic agent from the first cells lysed.

A study of many lysing colonies of pyocyaneus leaves little doubt that the L strain of pyocyaneus represents a culture in a delicate equilibrium. Large areas are constantly, so to speak, on the verge of lysis, and a small thing may precipitate the dissolution. One of these precipitating factors is merely bringing a fragment of culture into contact with fresh medium. For instance, let us observe an artificial colony on the threshold of lysis. It is apparently normal, homogeneous, without visible lytic areas. Yet we know that, within an hour perhaps, it will show a few lytic areas; and we know that we can at any time obtain a lytic agent from any square millimeter of that colony. We touch a needle to any unlysed part of the colony and then touch a sterile plate. Growth starts at once, but lysis also starts at once; so that within a few hours we have a colony showing a ring of active growth with a lysed center. In this instance it is clear that the transfer to fresh agar of cultural material on the threshold of lysis stimulated at once the lytic action through all the transferred culture and for a considerable distance, and many bacterial generations, beyond; then "recovery" took place. This situation is doubtless analogous to the "rejuvenating" or

"regenerating" effect of fresh broth on lysis, as reported by Bordet and Ciuca for transmissible lysis of B. coli cultures. It stimulates into immediate lysis many organisms which otherwise would not autolyze for a considerable time, if at all.

But it is also clear that for a medium to produce such regenerating effect on the lytic action, it must be so constituted that it not only keeps the bacteria on the way in which they are headed, but also gives them a violent push in that direction. The thing that gives the push may be some particular substance in the medium, or it may be a combination of substances, accompanied by a favorable reaction. It may be that the reason why bacteria suspended in salt solution do not undergo lytic action in the presence of a lytic agent is because the lytic agent by itself alone cannot give the necessary push unless the machinery of the cell is running; and the machinery of the cell cannot run unless the physical and the nutritive environment of the cell is such as to stimulate active metabolism and growth, accompanied by further generation of the lytic agent. It is this situation which must lead one to the view that the bacteria ultimately must be self-destructive.

Relation of the Lytic Agent to the Lysogenic but Nonlysing Cultures (R Type 1).—But there is another possible relation between the lytic agent and the bacterial organism that requires consideration, namely, the relation to the organisms in those pyocyaneus colonies which never present evidence of lysis, yet which may serve as a source of the lytic agent for a considerable time. In this case the colony develops only medium size, is always homogeneous, nonpigmented, except for the yellow-green pigment, does not produce crystalline bodies in fresh agar, is depressed with respect to the performance of all its enzymatic reactions, and reproduces only the nonlytic type of colony on subplating. How is the lytic agent maintained in such cultures of the R type? If one could discover among hundreds of subcolonies from such a culture even one that showed the characteristics of the lytic strain, the problem would be answered; but such lysing colonies have not been found.

One answer that can be given is that the organisms of the R type harbor the lytic agent through generation after generation of cells. If the lytic agent is regarded as parasitic on these cells, it plays the rôle of a commensal. The organisms have become, so to speak, "carriers"; they are not harmed by it, but on the other hand, they cannot eliminate it. While it is present, however, although not weakening them in growth vigor (compared with "normal" organisms), it exercises a pro-

found repressive influence on all their physiologic reactions. This view of the matter is attractive, and in harmony with many of the facts. It is, however, scarcely tenable.

Relation Between the Lytic Agent and Sensitive Cultures (S Type).—Finally, we must consider the relation between the lytic agent and the cells of quite a different kind of culture represented in the present study by the sensitive Mulford and the Wisconsin strains. When a few drops of the anti-Wisconsin L filtrate are added to a 12-hour Wisconsin culture in broth, and the tube is incubated for about 30 minutes, it is found on plating that, compared with a control, the number of organisms in the filtrate culture is greatly reduced, while those that appear on the plate seem to be of one kind. They are heavy, fleshy, convex, homogeneous and show only a yellow pigment in the surrounding medium. What is more, they show no evidence of harboring the lytic agent. When again brought into contact with the lytic filtrate and plated, these characters become even more pronounced, and even the yellow pigmentation may be lost, together with other typical pyocyaneus features. In this form they may be perpetuated for numerous generations without reversion.

In this case one is led to assume that the action of the pyocyaneus lytic agent is more like that of the d'Herelle filtrate against Shiga: The most sensitive organisms are destroyed quickly, while those that remain are of a more resistant type. But it is clear that between the resistant Wisconsin strain and the R strain heretofore referred to there are marked differences. While the original R strain carries the lytic agent and will give rise to potent lytic filtrates, the lytic agent is not obtained from the R Wisconsin strain; and it may be added that the same is true of the R Mulford.

Briefly to summarize the last few sections, it is pointed out that although transmissible lysis of pyocyaneus presents several differences, the fundamental facts are the same as in the case of lysis by the bacteriophage of d'Herelle. Any explanation of that phenomenon must also be able to explain these differences observed in pyocyaneus. To be more specific, the following points must be considered:

(1) The "carrying power" of apparently normal culture for the lytic agent, without evidence of lysis.

(2) The circumstance that some unknown factor found in a sterile culture medium is able to precipitate suddenly the lytic reaction in a culture mass that rests on what has been called the "threshold of lysis."

(3) The growth-stimulating effect of the presence of "carried" lytic agent on a bacterial mass approaching the "threshold of lysis," but not on an R type culture.

(4) The varying aspects of lytic action observed in different strains of pyocyaneus submitted to the influence of the lytic agent, or developing "spontaneous" lysis.

Various Types of Pyocyaneus Cultures.—From the foregoing data it appears that among the cultures of pyocyaneus studied, at least 5 differing types may be recorded.

(1) A lysogenic strain (referred to in the preceding pages as the lytic or the L strain): A pyocyanigenic and fluorescigenic strain characterized by spontaneous colony lysis and by its ability to give rise to the renewed lysogenic strain and also the strain next in order (L type I).

(2) A resistant strain, potentially lytic (referred to in the previous pages as the nonlytic, the resistant or the R strain): A nonpyocyanigenic, but fluorescigenic characterized by the absence of colony lysis, by its lack of ability to reproduce any but its own type, but also by its ability to yield filtrates possessing marked lytic action for sensitive strains (R type I).

(3) A sensitive strain, potentially nonlytic (referred to in the preceding pages as the Wisconsin and the Mulford strains): A pyocyanigenic (originally) and fluorescigenic strain characterized by the absence of spontaneous lysis, by great susceptibility to the action of lytic filtrates and by its ability to give rise to type 4 mentioned below (S type).

(4) A resistant strain, potentially nonlytic (referred to as the resistant Wisconsin strain): A nonpyocyanigenic, but fluorescigenic strain characterized by the absence of colony lysis, its inability to reproduce any but its own type, and by its inability to yield lytic filtrates (R type II).

(5) A lysogenic strain (referred to in the preceding pages as the Detroit strain): A nonpyocyanigenic, but fluorescinogenic strain characterized by spontaneous colony lysis and by its ability to give rise to the renewed lysogenic strain (L type II).

To these might be added a sixth type which lacks both pyocyanogenic and fluorescinogenic power. In view of Jordan's interesting results on pigment production of pyocyaneus, and his suggestion for the recognition of several varieties, the matter of chromogenesis in the types mentioned above may be briefly referred to again, although this phase of the problem is receiving further study and will be reported on later. Jordan recommends considering as the A variety those cultures which produce both pyocyanin and fluorescin; as the B variety those rare cultures, such as his "Rush," that produce pyocyanin only; as the C variety those strains that produce fluorescin only, which are closely related to B. fluorescens; and as the D variety those which are nonchromogenic.

Of the types that I refer to, the lysogenic or L type (1) produces a strong pyocyanin and would correspond with Jordan's B variety

except for the circumstance that it also produces fluorescin, which Jordan's B variety does not produce. My resistant or R type I (2) produces fluorescin only and would correspond with Jordan's C variety. My sensitive or S type (3) although originally pyocyanigenic now produces only fluorescin so far as observed and would also correspond with Jordan's C variety, although this point is still being studied. My type 4, and R type II differing from type (2) in that it does not "carry" the lytic agent, also corresponds with Jordan's C variety, although it is capable of losing all chromogenic power under further stimulus by the lytic agent, and of being transformed into Jordan's D variety.

The Relation of Pyocyanin Production to Lysis.—The problem of the control of pigment production has been approached from many angles, and although a variety of agencies which may influence the phenomenon have been established, little is definitely known regarding the part played by chromogenesis in the physiology of the bacterial cell. Pigment production by pyocyaneus has received careful study by Jordan (1899), and several component factors have been analyzed, but the difficulty in obtaining concise knowledge of the subject is increased because pigment production in pyocyaneus is highly variable compared with that of most other chromogenic bacteria. In some strains the power of producing either pyocyanin or fluorescin is lost quickly after isolation, although fluorescin production is the more stable. In other strains, although more rarely, the power of producing pyocyanin is maintained for many generations, and cultures which have lost this power have usually been termed "degenerate," since in the original type strain of Gessard the ability was present in considerable degree. Gessard (1891) was the first to show the possibility of establishing new "races" of pyocyaneus as a result of subjecting the cultures to varying conditions of growth. He was able to transform a race yielding both pigments into one producing only fluorescin by the action of heat or by passage through animals. He could also create a pyocyanigenic race by growing on egg albumin. Jordan, however, by using the same, and also other means, was unable to modify pyocyanin production except possibly by growth in ammonium tartrate solution in which no pigment was produced. In ammonium succinate, however, the pigment returned in the first generation. Jordan found pyocyanin production was not dependent on the presence of either sulphate or tartrate in the culture medium, although the absence of these substances

caused an inhibition of fluorescin production. Without considering further at this time the mass of work that has been done on the subject of the production of blue-green pigment in culture, it may be said that the conditions which control it are not yet recognized.

It is quite clear, however, that among possible modifying conditions one must take into consideration the lytic agent. While the statement that an energetic production of pyocyanin is dependent on the presence of a lytic agent in the culture is as yet scarcely warranted, the fact remains that among the cultures that I studied (and it may be added, in all probability among those studied by Jordan), there was none capable of intense pyocyanin production which did not carry, at least potentially, the lytic agent, although there was one lysogenic strain (Detroit) that was nonpyocyanigenic. The matter may be summed up for the present by saying that pigment production by pyocyaneus is doubtless dependent on many factors, but among these henceforth must be included the lytic agent which not only influences chromogenesis to a marked degree, but evokes other profound modifications in the physiologic activity by pyocyaneus cultures.

In observing the changes in bacterial type that are determined by the lytic action, one is tempted to speculate as to how extended may be the rôle that this agent plays in bacterial transmutation. There are many instances in which the R type, surviving lytic action, scarcely resembles the original strain. A recent interesting example of this is the modification of B. coli into a form closely resembling Bact. aerogenes; and there are many other instances.

In the case of pyocyaneus the study of Blanc [10] (1923), in which a lytic agent was undoubtedly involved, and the studies of Jordan, as well as the present investigation, all indicate with what justification we might regard the old B. fluorescens nonliquefaciens as a variety of pyocyaneus which has been modified physiologically as a result of contact with a lytic agent.

The "Spontaneous" Origin of a Lytic Agent from "Normal Cultures" and from Microbial Associations.—Within the past year many investigators have reported a "spontaneous" origin of a lytic principle from "normal" cultures or from a mixture of two bacterial species (so-called "bacterial antagonisms"). These reports usually purport to show that the lytic agent is derived "from the bacteria themselves." Sometimes this has been accomplished by use of old broth cultures, sometimes by use of young agar cultures. Of the former, one of the

most recent reports is that of Kuttner[14] (1923) who obtained a lytic agent from an old typhoid culture. This culture, however, came originally from a case in which the feces were shown to contain an active bacteriophage, and the culture was noted to have become resistant to lytic action before the lytic agent in it was discovered. A lytic agent was not obtained from a culture of a susceptible variant.

The isolation of a lytic agent assumed to have been developed as a result of antagonism in microbic associations has also been reported several times, and studied particularly by Lisbon and Carrere[15] (1922) and by Paul Farby[16] (1922), the former working with a combination of B. coli and Shiga dysentery, the latter with a combination of B. coli and staphylococci.

In all of these cases the question must arise as to what constitutes a "normal" culture. If such a culture is regarded as one in which there are no superficial evidences of lytic action, a dangerous fallacy is involved, for nothing is more certain than that the appearance of a culture, through no matter how many successive generations, cannot be employed as a criterion of its freedom from the lytic agent. D'Herelle's criticism of the conclusions of Lisbon and Carrere may be well founded: they were working with cultures which harbored the lytic agent. This view is supported by experiments of Bordet and Ciuca who demonstrated that a nonlytic colon culture might carry the lytic principle. Although Kuttner's earlier work with typhoid led her to the conclusion that "only the colonies in which she could definitely see evidences of lysis (i. e., lytic colonies) carried the virus," Kuttner now recognizes the uncertainty of such a conclusion, particularly in the case of freshly isolated cultures.

The data presented in the foregoing pages serve to support d'Herelle's criticism of the conclusions of Lisbon and Carrere,[15] and also parallel the observations of Bordet and Ciuca[17] and others with reference to the fact that the lytic agent may be carried by apparently "normal" cultures. The pyocyaneus R type harbored the agent through numerous cultivations without at any time lytic action being manifested for the homologous culture. These results justify the conclusion that it may be exceedingly difficult to interpret the facts involving the acquisition of a lytic agent from "normal" cultures. If we mean by the term "normal" merely apparent freedom from lytic manifestations,

[14] Jour. Bacteriol., 1923, 8, p. 49.
[15] Compt. rend. Soc. de biol., 1922, 86, p. 569.
[16] Ibid., 1922, 87, p. 369.
[17] Ibid., 1920, 83, p. 1923.

the recovery of a lytic agent from such a "normal" culture possesses no more significance in illuminating the problem of the source of lytic action than if the lytic agent had been derived from a frank lysogenic strain.

SUMMARY

This paper deals with the study of transmissible lysis of a culture of Bacillus pyocyaneus obtained from a suppurating lesion on the forehead of a private patient.

From this primary culture were obtained by plating on agar two different strains: (1) a lytic or lysogenic strain (L type): (2) a resistant, nonlytic strain (R type).

The L strain was characterized as follows: colonies, leaf-like and expansive with fimbriate margins, highly pyocyanigenic, but producing also fluorescin, and containing one or more roughly circular, corroded or pocketed areas, measuring from 1 to 5 mm. in diameter, in which bacteria had largely disappeared, leaving the surface bronze colored or iridescent; agar slant cultures present similar features, the lytic areas being limited to middle and lower portions of the tube; possessing remarkable growth energy in broth and on agar; highly proteolytic with production of indol; medium grade of virulence for guinea-pigs; on plating, giving rise to colonies of both the L and the R type.

The R strain was characterized as follows: colonies round, of limited size and somewhat massive and highly viscous growth, homogeneous in texture, forming only the yellow pigment and no pyocyanin; similar homogeneous growth on agar slants with no lytic pockets; slight or delayed proteolytic action on milk and gelatin; more virulent than the L strain for guinea-pigs; giving rise on plating to R type colonies only.

From both the L strain and the R strain were obtained filtrates which produced weak inhibition and lysis of sensitive strains of pyocyaneus, but not of either of the homologous strains, which grew luxuriantly in their own filtrates.

The lytic power of the L filtrates could be increased by feeding with sensitive strains of pyocyaneus to give a maximum observed inhibition titer of 2×10^{-6}; the lytic power could not be increased appreciably by feeding an L filtrate with the L or the R strains, or by feeding an R filtrate with the same strains. Filtrates from two S strains were not lytic.

The pyocyaneus L filtrate in high dilution applied to agar slants simultaneously with cultures of sensitive cultures gave lytic "colonies"

which were numerically in proportion to the filtrate dilution or to the amount used. The addition of similar L filtrate to L culture did not increase the number of lytic colonies; and added to R culture did not produce lytic colonies.

When L filtrate acts on sensitive cultures (S type), all the bacteria are not killed, but organisms resistant to lysis remain in the culture. These resistant organisms perpetuate a resistant strain, and this strain is characterized by partial or entire loss of blue-green pigment production, proteolytic power and motility; it may or may not continue to produce fluorescin, and resembles B. fluorescens nonliquefaciens.

The reaction of pyocyaneus cultures to the lytic agent affords the basis for presenting at least 5 different types, A B C D and E, each of which differs from the others with respect to one or more of the following characteristics: (1) production of pyocyanin; (2) production of fluorescin only; (3) production of neither; (4) ability to perpetuate in subculture the lytic agent accompanied by frank lysis; (5) ability to perpetuate in subculture the lytic agent without manifestation of lysis; (6) susceptibility to the lytic agent.

It is shown that the lytic agent has a marked influence on the production of pyocyanin. While pyocyanigenic cultures may belong to either the L or the S type, the R type is always nonpyocyanigenic and may also be nonfluorescigenic (achromogenic).

The L filtrate showed some inhibitive action on B. anthracis and B. fluorescens, but not on Shiga dysentery, B. coli, or on the common chromogens. D'Herelle anti-Shiga and anticoli bacteriophage from various sources, and active for intestinal bacteria, did not show lytic action on pyocyaneus cultures after three or four serial passages.

The L filtrate is rendered inactive by heating at 76 C. for 30 minutes. The same temperature, but not a lower temperature, also destroys the protease which is able to digest gelatin without the aid of living organisms. At temperatures of 70 to 74 C. both are only partially destroyed.

When sterile, unheated L filtrate is added to a tube of solidified gelatin, and the gelatin has become partly liquefied as a result of the action of the protease, the fluid portion no longer contains active lytic agent for sensitive pyocyaneus cultures. Melted gelatin in similar dilutions, added to L filtrate, does not cause inactivation.

While the study was under way a similar lytic action was observed in three other laboratory strains of pyocyaneus which had not previously

shown evidence of lysis. Although in the main, similar to the first case, they presented several differences in the mode of lysis and in the results.

Since it has been shown that pyocyaneus organisms may "carry" the lytic agent for hundreds of bacterial generations without giving any manifestation of lytic action in the culture mass, it is concluded that the reported isolations of lytic principle "from the bacteria themselves," or from the "bacterial antagonisms" possesses little more significance than similar isolation of lytic agent from frank lysogenic strains.

The lytic phenomenon described for pyocyaneus closely resembles the lysis of staphylococcus cultures described by Twort. In its most essential features it also resembles the transmissible lysis of d'Herelle and of Bordet and Ciuca, from both of which it differs mainly in the speed with which the reaction occurs and in the greater complexity of the modifying factors. It is concluded that the Twort phenomenon, the d'Herelle phenomenon and lysis in pyocyaneus are all dependent on the same fundamental cause.

In the lysis of pyocyaneus cultures on agar it appears that the process involves two stages: (1) the gradual approach of the mass of organisms harboring the lytic agent to what has been termed the "threshold of lysis"; (2) the precipitation of the actual lytic action by some unrecognized factor of the environment, with the consequent production of circumscribed lytic pockets and broad erosions.

In any attempted explanation of transmissible lysis in general, it is regarded as a point of special significance that in pyocyaneus lysis the behavior of the lytic agent and its fluctuations in energy go hand in hand with fluctuations in other zymatic activities of the cell. This fact suggests that the thing which effects ultimate lysis is not a substance foreign to the cell, but a normal part of the cell organization which becomes stimulated into intensified action under the influence of a still unrecognized factor in the environment; the bacteria are self-destructive.

Since lysis of pyocyaneus is a "low speed" reaction in which successive stages can be followed more closely than in the lysis of Shiga and coli cultures by the d'Herelle bacteriophage ,this phenomenon may be a more favorable subject for study of some of the detailed features of transmissible bacterial autolysis than is lysis in most other bacterial species.

PLATE 1

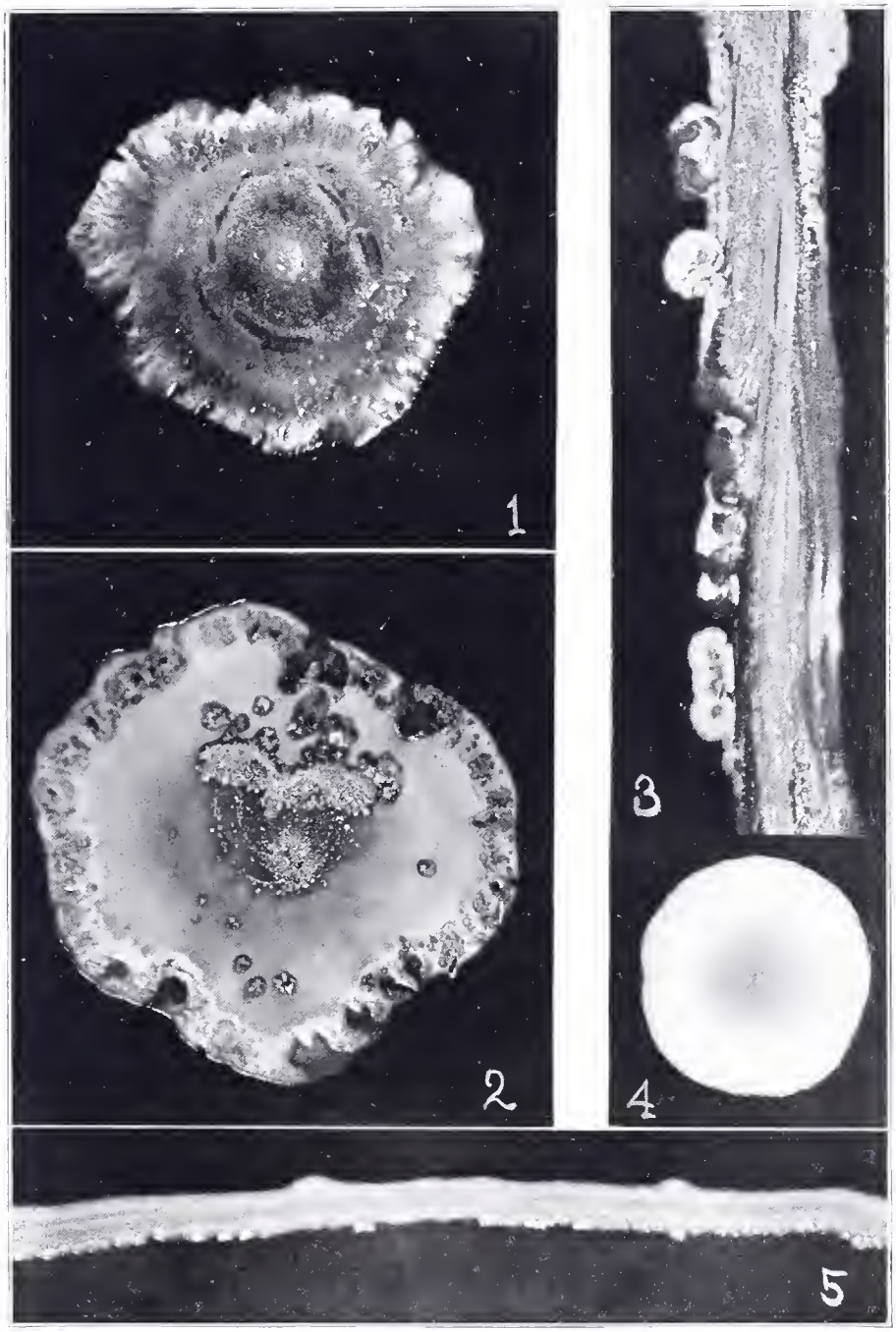

Fig. 1.—Lytic colony A on infusion agar, 3 days old. Showing lytic pockets and zonal areas of mass lysis. × 1.2.

Fig. 2.—Lytic colony B on infusion agar, 4 days old. Showing lytic pockets, mass lysis and marginal lysis; also crystalline deposits in the center of several pockets. × 1.0.

Fig. 3.—Streak growth of lytic culture, 5 days old. Showing marginal lysis and small pockets; also the expansive growth compared with Fig. 5. × 1.2.

Fig. 4.—Resistant colony on infusion agar, 4 days old. Showing smooth, homogeneous surface. × 1.0.

Fig. 5.—Streak growth of resistant culture, 5 days old. Showing even growth free from lytic changes; also restricted growth compared with Fig. 3. × 1.2.

PLATE 2

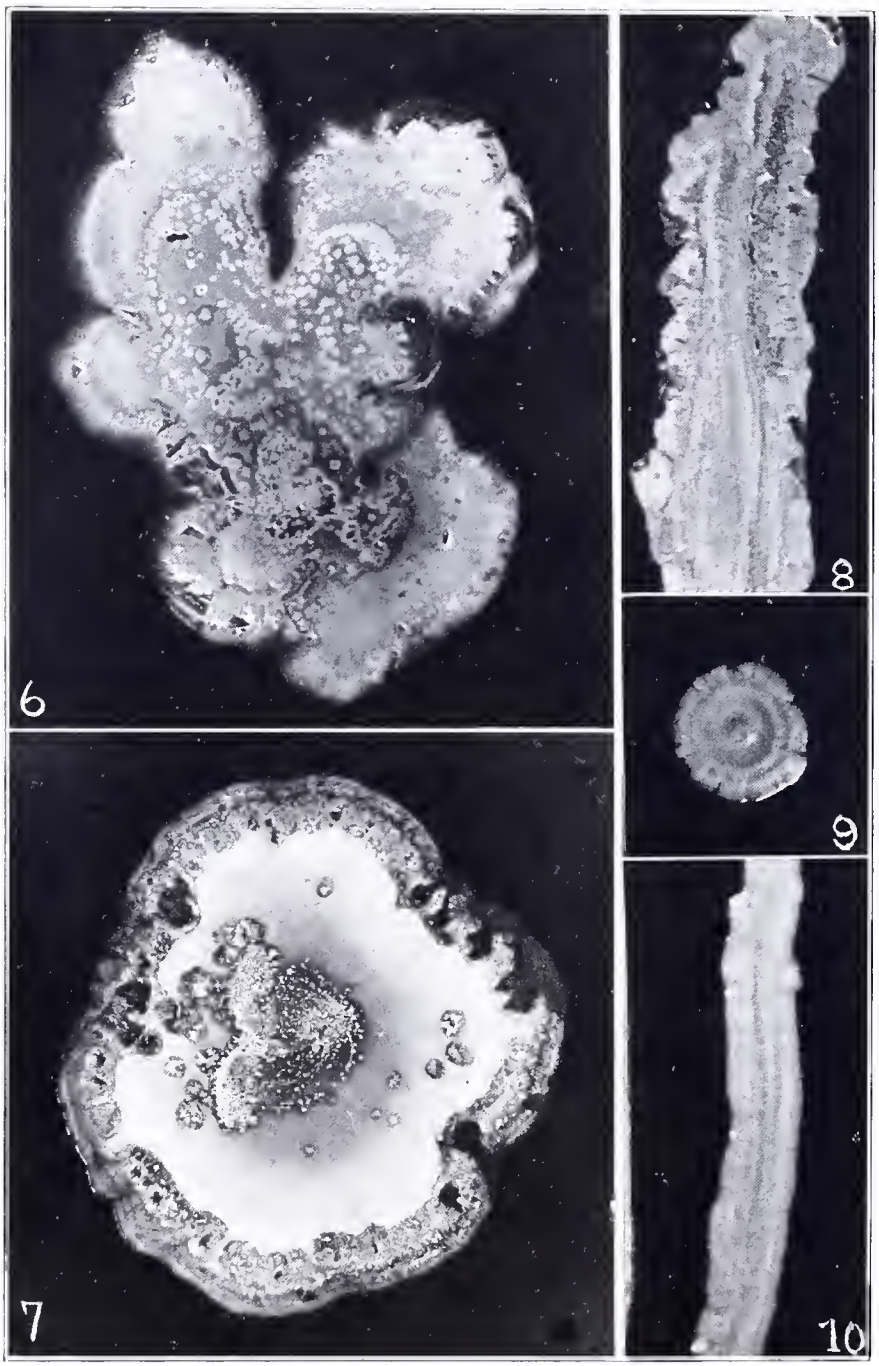

Fig. 6.—Lytic colony C on infusion agar, 5 days old. Showing the marginal rings around the lytic centers; also the beginning of marginal lysis. × 1.0.

Fig. 7.—Lytic colony B on infusion agar, 7 days old. Showing mass lysis, lytic pockets and marginal lysis well under way. Crystalline deposits may be seen inside some of the isolated pockets. × 1.0.

Fig. 8.—Streak growth of lytic culture, 4 days old. Showing beginning of lysis and the beginning of expansive growth. × 1.6.

Fig. 9.—Colony of resistant strain on infusion agar. Showing modification probably due to the inclusion of lytic agent. × 1.0.

Fig. 10.—Streak growth of resistant culture, 4 days old. Showing absence of lytic areas, and less extensive growth than in Fig. 8. × 1.6.

PLATE 3

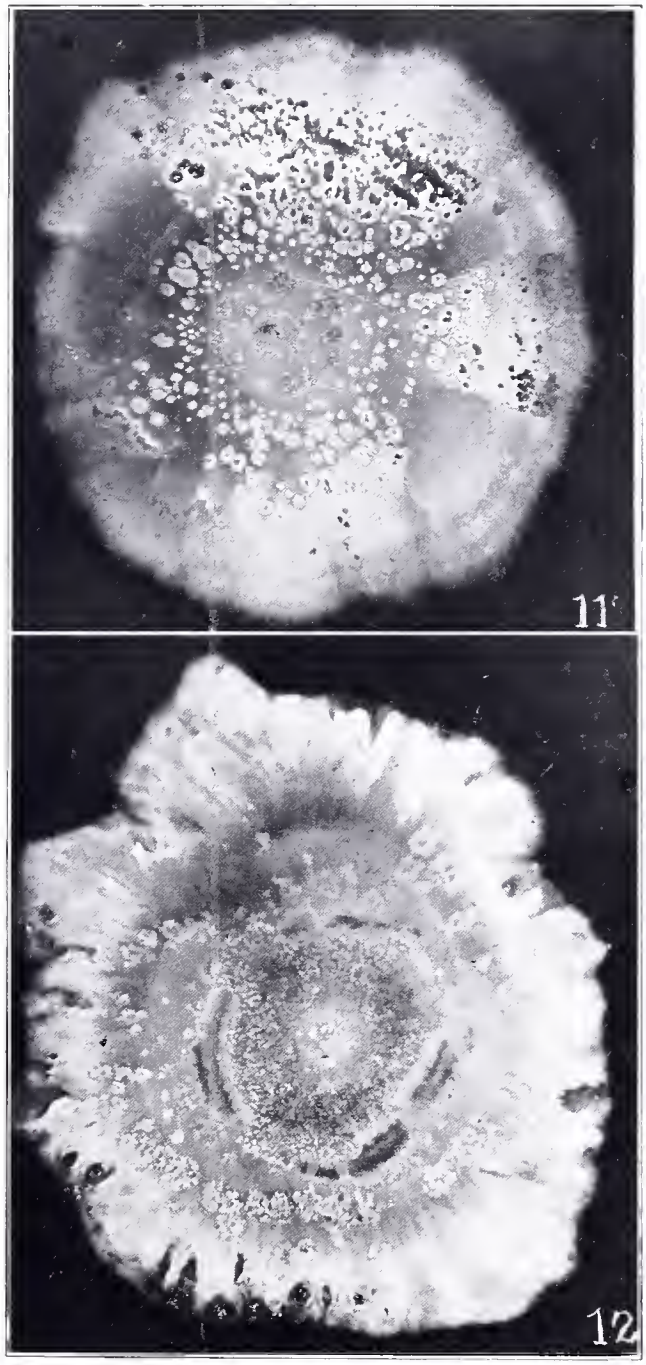

Fig. 11.—Lytic colony D on infusion agar, 8 days old. Showing radial and concentric zones of lysis, also the ringed lytic pockets and crystalline deposits. × 1.0.

Fig. 12.—Lytic colony A on infusion agar, 8 days old. Showing zonal areas of lysis and many lytic pockets. × 1.2.

AGGLUTININS FOR PFEIFFER BACILLUS IN SERUM OF INFLUENZA AND MEASLES PATIENTS

INFLUENZA STUDIES. XVII *

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While some observers [1-15] have found specific agglutinins for the Pfeiffer bacillus in the serum of influenza patients and convalescents, others [16-18] have failed to obtain positive results. Skajaa [19] found Pfeiffer bacillus antibodies in the serum of influenza patients only exceptionally. Fichtner [20] and Ghedini [21] observed contradictory and confusing reactions with control normal serum so that they attached little importance to the agglutination reactions sometimes produced by the serums of influenza patients. Nearly all recent observers, however, are agreed that the serum of healthy persons not recently attacked by influenza and also that of persons suffering from other diseases have little or no agglutinative power for the Pfeiffer bacillus.[1, 3, 6, 7]

Our own observations were made on the serums of 29 influenza patients, of 29 measles patients and of 27 other persons. The influenza

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serums were obtained during the January-February, 1920, epidemic of influenza from patients at the Great Lakes Naval Training Station (table 1, "G L" series), at Camp Grant, Rockford, Ill. (table 1, "R" series) and at Chicago (table 1, "C" series). The measles serums were obtained in December, 1922, and January, February and March, 1923, from patients at the Cook County Hospital, Chicago, through the kindness of Dr. F. B. Kelly. The control serums were obtained in January-March, 1921, and June, 1922, from 12 healthy students of the University (table 1, "B and D" series), and from 15 noninfluenzal patients at the Cook County and Presbyterian Hospitals.

The influenza and measles serums were frozen, and stored in the freezing box for some months before the tests here recorded were made. The control serums used for comparison were fresh. Our experience with Pfeiffer bacillus serum from immunized animals which was frozen and stored under the same conditions has shown that such change as occurs, although slight, is in the direction of a diminution of agglutinative power. It does not seem likely therefore that the agglutinative power of the human influenza serum was increased by storage, and it is probable that the real difference between the reactivity of the influenzal and the measles serums on the one hand and that of the noninfluenzal serums on the other was somewhat greater than that shown in the tables.

The tests were made with serums in 1 : 50 dilution. The strains used were mainly those described in a previous paper.[22] They included strains from the same influenza cases from which the serum was obtained, from other cases occurring in the same local outbreak (GL series, R series, etc.), and from still other cases occurring in different localities (New York and Chicago) ; strains from the throats of normal persons and of those suffering from various types of common colds were also tested. The new strains used in place of a few that had dried out did not appear any different in their general reactivity. As in the previous experiments, the mixtures of serums and bacterial suspensions were incubated in the water bath at 55 C. for two days.

The results are given in table 1.

While the agglutinative power of the serum from influenza patients is usually not very high and is never so high as that of immune animal serums, there is no doubt from these observations that on the average it is greater than that of the serum from persons not having influenza. The proportion showing complete agglutination at 1 : 50 dilution is

[22] Jordan and Sharp: Jour. Infect. Dis., 1922, 31, p. 198.

noticeably greater with the influenza serum than with the other serums. More than half of the influenza serums completely agglutinated one or more strains as against about one-fifth of the control serums, and the influenza serums completely agglutinated 7.5% of all test suspensions as against 0.7% agglutinated by control serums. Comparison of partial

TABLE 1

AGGLUTINATION OF THE PFEIFFER BACILLUS WITH SERUM FROM

Influenza Patients					Controls*				
Serum 1:50 Dilution	No. of Strains Tested	Reactions			Serum 1:50 Dilution	No. of Strains Tested	Reactions		
		Complete	Partial	Negative or Trace			Complete	Partial	Negative or Trace
GL 1	47	16 (34%)	15 (32%)	16 (34%)	B 1	31	0	8 (26%)	23 (74%)
GL 3	38	1 (3%)	7 (18%)	30 (79%)	B 2	42	0	0	42 (100%)
GL 4	36	1 (3%)	7 (19%)	28 (78%)	B 3	35	0	3 (9%)	32 (91%)
GL 6	44	1 (2%)	20 (45%)	23 (53%)	B 4	40	0	0	40 (100%)
GL 7	34	0	8 (24%)	26 (76%)	B 5	37	0	5 (14%)	32 (86%)
GL 8	44	0	1 (2%)	43 (98%)	B 6	39	0	2 (5%)	37 (95%)
GL 9	47	0	4 (9%)	43 (91%)	B 7	32	0	1 (3%)	31 (97%)
GL 10	21	0	3 (14%)	18 (86%)	B 8	32	1 (3%)	0	31 (97%)
GL 11	42	3 (7%)	8 (19%)	31 (74%)	D 1	39	0	2 (5%)	37 (95%)
GL 12	22	1 (4%)	7 (32%)	14 (64%)	D 2	39	0	4 (10%)	35 (90%)
GL 13	42	3 (7%)	9 (21%)	30 (72%)	D 3	36	0	3 (8%)	33 (92%)
GL 14	43	16 (37%)	12 (28%)	15 (35%)	D 4	33	0	0	33 (100%)
GL 15	44	9 (20%)	11 (25%)	24 (55%)	Pn 1	30	0	7 (23%)	23 (77%)
GL 16	31	0	2 (6%)	29 (94%)	Pn 2	30	1 (3%)	4 (13%)	25 (83%)
GL 18	47	0	5 (11%)	42 (89%)	Pn 3	40	0	9 (22%)	31 (78%)
GL 20	31	0	2 (6%)	29 (94%)	Pn 4	40	1 (2%)	3 (7%)	26 (90%)
GL 21	43	0	1 (2%)	42 (98%)	F 1	30	1 (3%)	3 (10%)	26 (87%)
R 1	31	1 (3%)	3 (10%)	27 (87%)	F 2	40	1 (2%)	10 (25%)	29 (72%)
R 2	5	0	1 (20%)	4 (80%)	F 3	40	0	10 (25%)	30 (75%)
R 3	43	6 (14%)	23 (53%)	14 (33%)	F 4	40	1 (2%)	6 (15%)	33 (82%)
R 4	38	3 (9%)	4 (10%)	31 (81%)	P 1	34	0	1 (3%)	33 (97%)
R 5	37	7 (19%)	14 (38%)	16 (43%)	P 2	35	0	0	35 (100%)
R 6	41	1 (2%)	6 (15%)	34 (83%)	P 3	36	2 (6%)	7 (19%)	27 (75%)
R 9	14	0	0	14 (100%)	P 4	33	0	0	33 (100%)
R 10	33	3 (9%)	6 (18%)	24 (73%)	P 5	36	0	0	36 (100%)
R 11	33	0	4 (12%)	29 (88%)	P 6	35	0	1 (3%)	34 (97%)
R 12	7	0	1 (14%)	6 (86%)	P 7	40	0	5 (13%)	35 (87%)
C 82	8	0	0	8 (100%)					
C 627	8	0	2 (25%)	6 (74%)					
	954	72 (7.5%)	186 (19.5%)			1060	7 (0.7%)	100 (9.4%)	

* Controls selected are: B 1-6, healthy persons without history of influenza; D 1-4, healthy persons with history of influenza during epidemic periods; Pn 1-4, pneumonia cases, Pn 1 bronchial and the rest lobar, with previous influenza history; F 1-4, cases with fever, nonrespiratory, without history of influenza during the epidemic periods; P 1-7, unselected hospital patients not suffering from influenza.

agglutinative reactions shows a similar, but not so great, difference; 19.5% of all test suspensions were partially agglutinated by influenzal serums as against 9.4% of control serums.

Table 1 also shows clearly that the influenza serums were possessed of different degrees of reactivity. Our observations do not reveal the reason for this difference. The two serums of greatest agglutinative power were derived respectively from a patient near the end of a fatal influenzal pneumonia and from one in the second day of the disease.

Twelve of our 29 serums were obtained from patients during the first 3 days of the disease and 17 from patients 7 to 22 days after onset. Five of the former and 4 of the latter manifested the strongest agglutinative power. Serums drawn from patients at one stage of the epidemic did not possess any greater agglutinative power than those drawn at another.

The reactions of all serums are briefed in table 1. For further comparison the detailed reactions of certain serums which completely agglutinated more than a single Pfeiffer bacillus strain are given in table 2.

Perhaps the most noticeable feature of table 2 is the difference there shown in the sensitiveness of different strains of the Pfeiffer bacilli to agglutination by patients' serums. Some strains are agglutinated by practically all 9 serums (for example, 026, a meningitis strain isolated in Chicago before the 1918 influenza epidemic; GL 7 NP ba, a strain from the nasopharynx of an influenza patient in January, 1920; C 20 No a strain from the nose of a patient with a common cold in Chicago in December, 1919).

The results reported in table 2 were obtained with 5 serums obtained from influenza patients at the Great Lakes Naval Training Station (January, 1920) and 4 from influenza patients at Camp Grant, Rockford, Ill. (January, 1920). Inspection of the table shows that some of the Rockford serums agglutinate the Great Lakes strains as strongly as do the Great Lakes serums (e.g., GL 7 NP ba). The converse is also true (R 1 NOoa). Some of the serums manifest strong agglutinating power for a large number of the Pfeiffer bacillus strains (GL1), while others (GL 13, R 10) agglutinate relatively few strains. It is especially striking that strains of Pfeiffer bacilli isolated at distant points, at widely separated time intervals, from normal persons and from persons suffering from various throat infections are agglutinated as strongly as are those isolated from influenza cases in the same local influenza outbreak supplying the particular patient's serum. It is noticeable also that strains obtained from the patient yielding the serum do not always agglutinate with the homologous serum as strongly as they do with serums from other sources (G L1—2 T oa; GL 13). Finally, different strains of Pfeiffer bacilli isolated from one and the same patient at the same time show serologic differences (e.g., GL 6 and R 1).

Although our observations leave no doubt that Pfeiffer bacilli are agglutinated by the serums of influenza patients in a relatively high

TABLE 2

AGGLUTINATION BY THE MOST REACTIVE OF THE PATIENTS' SERUMS *

Test Strains		Test Serums of Influenza Patients										
		GL 1		GL 11	GL 13	GL 14		GL 15	R 3	R 4	R 5	R 10
Source of Isolation	Designation	10th# Day	Dilution	3d Day	2d Day	2d Day	Dilution	11th Day	16th Day	3d Day	3d Day	14th Day
Influenza, Chicago, 1918	O 6	+++	50	0	0	+++	50	+++	tr.	0	++	
Influenza (lung)	O 7	+++	50	0	0	+++	50	+++	+	0	0	0
Normal throat	O 13	+++	...	tr.	+	+	...	+++	0	+	++	0
Isolated from influenza cases, New York City, during the pandemic of Sept.-Oct., 1918	O 20	+++	...	+	+	.....	...	.....	.....	+		
	O 21	+++	50	+	+++	++	...	++	+	+++	0	+
	O 22	.....	...	+	tr.	+++	100	+	+++	tr.	.....	+++
	O 24	+++	200	+++†	+++†	.....	...	+++	.....	+++†	tr.	
Influenzal meningitis, Chicago.	O 26	+	...	+++	+++†	+++	50	+++	+++	+++†	0	+++
Rhinitis, 14th	C 20 No	+++	100	++	++	+++	50	+	++	++	+++†	0
	C 20 NPba	++	...	tr	tr.	+++	50	+	+++	tr.	0	
Rhinitis, 20th	C 56 NP	+++	...	.....	.....	++	...	++	+++	.....	.....	+++
Normal throat	NC 93 NP	tr.	..	0	0	0	...	0	0	0	0	0
Rhinitis, 7th	C 225 NPb	+++	50	.....	.....	+++	50	0	++	.....	+	+
Normal throat	NC 228 T	0	...	0	0	0	...	.....	+	0	.....	0
Rhinitis, acute	C 229-2 b	++	...	0	0	0	...	0	0	0	0	0
Tonsillitis, 3d	C 238 NP	.....	...	0	0	0	...	0	0	0	tr.	0
Pharyngitis, 4th	C 241 NPb	++	...	0	0	0	...	0	0	0	++	0
Rhinitis, 6th	C 575 NPva	++	...	+++	+	.....	...	tr.	.....	+		
Influenza, 4th	IC 630 NPoa	tr.	...	0	0	+++	50	0	+	0	++	
	IC 630 NPoc	+++	50	0	0	0	...	0	++	0	0	0
Influenza, 4th	GL 1 Toc	+++	50	0	0	+++	50	+++	+	0	++	0
	GL 1 Tba	tr.	...	+	+	++	...	0	+	+	0	0
Same case, 10th	GL 1-2 Toa	0	...	0	0	++	...	0	+	0		
Influenza, 3d	GL 6 NPbe	+++	50	+	+	0	...	0	+	+	+	0
	GL 6 NPob	+	...	0	0	+	...	0	0	0	0	0
	GL 6 Toa	0	...	0	0	+	...	0	+	0	0	0
Influenza, 9th	GL 7 NPob	0	...	0	0	+	...	.....	0	0	0	0
	GL 7 NPba	+	...	+	+	+++	50	+++	++	+	+++†	++
Influenza, 3d	GL 10 Tbe	0	...	tr.	+	+++	50	0	++	+	+	0
Influenza, 4th	GL 13 Toc	+	...	0	0	+++	50	0	+	0	++	
Influenza, 4th	R 1 NPoa	+++	500	.....	.....	.....	...	0	.....	+		
	R 1 NPoc	tr.	...	0	0	.....	...	0	.....	0		
	R 1 Tbe	tr.	...	0	0	0	...	0	+	0	0	
Influenza, 15th	R 9 Toc	++	...	0	0	0	...	0	0	0	+	0
	N 9 NPob	tr	...	0	0	0	...	0	+	0	0	0
Normal throat	N 10 NPoa	++	...	0	0	+++	50	.....	+	0		
	N 10 NPob	++	...	.....	.....	++	...	.....	+++	.....	.....	++
	N 18 NPob	tr.	...	0	0	0	...	+	0	0	tr.	
	N 22 NPoa	+	...	.....	.....	tr.	...	0	++	.....	+	+
	N 38 NPva	0	...	.....	.....	0	...	+	+++	.....	++	+
	N 44 NPoa	tr.	...	0	0	0	...	0	0	0	++	0
	N 47 NPob	+	...	0	0	0	...	++	0	0	+	0
Throat infected	E 7-6 Tba	+++	100	.....	.....	++	...	+++	+	.....	+++†	0
	E 8-5 Tbe	0	...	0	0	++	...	0	0	0	+++†	0
	E 13 Tba	++	...	0	0	+++	...	+	0	0	+++	0
	E 16 Tba	+++	100	tr.	tr.	+++	50	+++	+	tr.	+++	
	E 24 Tba	++	...	.....	.....	++	...	+	+	.....	.....	0
Normal throat	NC 492-4 NPoa	0	...	tr.	0	.....	...	+	.....	0		
	NC 567 NPoa	+++	50	0	0	+	...	0	+	0	+++	

* These reactions are selected from those briefed in table 1.

† In some instances in which a complete agglutination reaction was obtained, the experiment was repeated and the results indicated by † when again complete, and by + when partial on repetition.

The figures indicate the number of days after onset.

proportion of cases, it does not follow that these bacilli bear any primary causal relation to true influenza.

Twenty-nine (29) serums from measles patients were tested with 38 strains of Pfeiffer bacilli. The results were similar to the results obtained with influenza serums, except that the percentage of complete agglutination in 1 : 50 dilution with the measles serums was higher than with the influenza serums, 7 of the measles serums completely agglutinating half or more of the strains tested, while (table 1) only 1 of the influenza serums completely agglutinated as high as 34% of the strains. Two of the measles serums failed to agglutinate any of the Pfeiffer bacillus strains, 2 agglutinated only 1 strain each and 5 others did not agglutinate more than 3. Two serums agglutinated, respectively, 35 and 32 of the 38 strains, and the others were distributed between the two extremes. Comparing these results with those obtained with influenza serums, as shown in table 1, it is clear that a considerably larger proportion of measles serums than of influenza serums completely agglutinated the Pfeiffer bacillus.

There was no demonstrable connection between influenzal history in the measles patients and the agglutination: 13 had had influenza, 10 gave no influenzal history, and 6 were doubtful. Of the 7 whose serums agglutinated half or more of the strains, 4 had had influenza, 3 had not. Of the 4 showing least agglutinative power, 2 had had influenza, 2 had not. Our tests with the measles serums again emphasized the advisability of using a number of strains in comparisons of this sort. There is almost as great a variation in the reactivity of different strains as in that of different serums. One of the 38 strains was agglutinated by only a single serum; another was agglutinated by 19 of the 29 serums.

It is tempting to suppose that the agglutinins for the Pfeiffer bacilli in the blood of both measles and influenza patients were due to a secondary invasion of the body with this organism, but our observations do not support this view. Pfeiffer bacilli were found on a single examination in the throats of a little less than one-half (13) of the 29 measles patients. There was no demonstrable relation between the presence of the Pfeiffer bacillus and the agglutinative power of the serum. Of the 7 serums that agglutinated half or more of the strains, only one was from a patient whose throat culture yielded the Pfeiffer bacillus. If throat findings are an evidence of Pfeiffer bacillus invasion, the agglutinative reaction must be nonspecific.

The most plausible inference from all these data is that both measles and influenza serums owe their higher reactive power, as compared with normal serums, to the presence of nonspecific antibodies. The few control serums (F1-F4) in table 1 that were obtained from hospital cases of nonrespiratory diseases with fever are plainly in line with this interpretation. We seem justified in concluding that the heightened agglutinative power of influenza serum for the Pfeiffer bacillus, as compared with normal serum, is not specific and does not necessarily connote even a secondary invasion on the part of this organism.

SUMMARY AND CONCLUSIONS

The serum of influenza patients agglutinates Pfeiffer bacilli to a greater degree and in a larger number of cases than does the serum of healthy persons.

The same is true, however, of the serum of measles patients, so that the presence of agglutinins for the Pfeiffer bacilli in influenza serum cannot be taken as evidence of any causal relation between this organism and influenza. There is no evidence that the observed increase in agglutinative power in measles serum is dependent in any degree on a secondary invasion by the Pfeiffer bacilli. The most plausible explanation seems to be that infections of various kinds increase the nonspecific or normal agglutinins of the blood, and that such relatively slight agglutination enhancement as is observed falls in this category and not in that of specific causal relation.

FIXATION REACTIONS WITH TYPES OF B. DIPHTHERIAE

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The experimental work of Durand,[1] Park,[2] Havens,[3] Langer,[4] FitzGerald and Doyle[5] and others has shown clearly the existence of several types of B. diphtheriae. These organisms may show no macroscopic or microscopic differences, one from another, and yet give rise to specific agglutination antibodies. There is, however, little literature on the specificity of the fixation bodies built up by animal immunization with these groups.

Studies on fixation reactions have been made, but have been for the most part unsuccessful, or at least have served only in differentiating between the true diphtheriae organisms and the diphtheroids. Such differentiation has been done by Kolmer,[6] Morse,[7] Armand-Delille[8] and others. The attempt to demonstrate by means of the fixation reaction the group specificity of diphtheriae organisms, as herein described, was made with groups of organisms isolated and identified by FitzGerald.[5] Taking representative strains from each of 3 groups, animals were immunized by repeated injections with washed cultures. These injections, beginning with 1/20 slant, were given about every other day unless the animal had lost too much weight. Dosage was increased until ½ slant could be administered. The cultures were grown on Loeffler slants for 24 hours, emulsified in salt solution and subjected to 8 washings. This was found necessary because of the extremely toxic effect of the organisms or rather their adherent toxins on the rabbits. With fewer washings of the cultures, the animals would appear well after one or two injections, but would then soon begin to lose weight and finally die. Tests of both the complement-fixing and agglutinating powers were made as immunization progressed, using however only the specific

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[1] Compt. rend. Soc. de biol., 1920, 83, p. 613.
[2] Jour. Immunol., 1922, 7, p. 243.
[3] Jour. Infect. Dis., 1920, 26, p. 388.
[4] Centralbl. f. Bakteriol., II., 1916, 78, p. 117.
[5] Jour. Am. Med. Assn., 1923, 80, p. 1675.
[6] Jour. Infect. Dis., 1912, 11, p. 44.
[7] Ibid., p. 433.
[8] Compt. rend. Soc. de biol., 1908, 65, p. 417.

organism with which the animal was being immunized. By these simultaneous tests it was evident that the rapidity of the building up of agglutinins and alexin-fixing bodies varied considerably in respect to the time of their appearance and the height of their reacting power. Table 1 is typical of the antibody response.

It may be of interest to note that, contrary to the results of some, the toxicity of the strains in respect to rabbits differed. Group 1, organisms apparently identical with the well-known "Park 8" strain, somewhat less toxic than group 2; whereas group 3 was, in comparison with the other two, extremely toxic, and immunization had to be carried out with great caution, many animals being lost during the process, even when the dosage was far smaller than in the other two series.

TABLE 1

RESULT OF IMMUNIZATION TESTS

	No. of Injections	Agglutination	Fixation
Rabbit 428 immunized with and tested against Group 1	3	1:320	0
	6	1:1,600	0.1 c.c. (weak fixation)
	13	1:3,200	0.0015625 (strong fixation)
	14	3,200	0.00078125 (fairly strong fixation)
Rabbit 433 immunized with and tested against Group 2	2	1:20	0
	5	1:320	0
	9	1:1,600	0.0015625 (strong fixation)
Rabbit 434 immunized with and tested against Group 3	2	1:25	0
	5	1:80	0
	9	1:3,200	0.003125 (strong reaction)

After the consideration of various modifications, the following fixation technic was adopted as the most satisfactory: Alexin (complement) and hemolytic sensitizer, 2 units, antigen ½ unit; the immune serum was used in decreasing amounts, controls of all dilutions, as well as normal serum and other standard checks, being used; the total volume in each tube was 2.5 c c.; antigen, alexin and serum were kept in contact for ½ hour at 37 C., and at the end of this time 0.5 c c. of 3% packed sheep cells + 2 units of hemolytic serum were added, and further incubation was carried out until the controls had cleared, or not exceeding ¾ hour.

Various types of antigen were prepared, as here briefly discussed.

1. Cultures grown 48 hours on Loeffler slants, washed off with 12 c c. salt solution; and this incubated 4 days at 37 C. This is antigen 1A. Part of antigen 1A was centrifugalized, and supernatant fluid known as antigen 1B. The precipitated organisms were washed 4 times and resuspended in the original volume of salt solution—antigen 1C.

2. Cultures grown 48 hours on Loeffler slants and then placed in ice chest for 4 days. Organisms washed off with 12 c c. of salt solution to each slant. This lot was also divided into 3 parts: 2A being whole antigen; 2B the supernatant fluid after centrifugalization; 2C the sediment of 2B washed 6 times, precipitated with alcohol and ether, dried, ground, resuspended (to original volume), and incubated 4 days before using.

3. The 24-hour cultures suspended in salt solution, washed 6 times, then treated with absolute alcohol, followed by absolute ether; desiccated and resuspended to original volume.

4. The 24-hour cultures washed off with salt solution and incubated 24 hours.

Without going into details as to the merits of these antigens, it may be stated that types 4 and 1A gave the best results, antigen 4 being slightly less anticomplementary and quicker of preparation. Antigens of the various groups were made and tested simultaneously. Table 2 is an example of the results obtained by the use of antigen 1A, B and C. The washed organisms (1C) it would seem have little, if any, antigenic power; this is also true of antigen 2C.

TABLE 2

RESULTS OF EXPERIMENTS WITH ANTIGENS 1A, B AND C

Amount					Fixation (Group 1)		
Serum Group 1 +	Antigen	+ Alexin +	Sensitizer	+ Cells	Antigen 1A	Antigen 1B	Antigen 1C
0.1 c c.	0.2 c c.	2 units	2 units	0.5 c c. 3%	+++	+++	0
	0.1	2 units	2 units	0.5 c c. 3%	+++	+++	0
	0.05	2 units	2 units	0.5 c c. 3%	+++	+++	0
	0.025	2 units	2 units	0.5 c c. 3%	++	+	0
	0.0125	2 units	2 units	0.5 c c. 3%	Trace	Trace	0
	0.00625	2 units	2 units	0.5 c c. 3%	0	0	0

When the serum of the animals gave a satisfactory fixation titer with their homologous antigens, they were bled, and subjected to test with all the antigens. It was found necessary to use both immune serums and the normal serum controls when quite fresh. On standing, even in the ice chest, both became highly anticomplementary. Using the same technic as in the trial fixations, many group interfixations were made. Table 3 illustrates the results obtained throughout. In all tests, 2 units each of alexin and sensitizer were used, with 0.5 c c. of 3% red cells and ½ unit of antigen, each made up to 0.5 c c. = total volume 2.5 c c.

While group specificity is apparent in the higher dilutions, the reaction is not nearly as simple to obtain as agglutination with the same serums, which, in this phenomenon, show no interagglutination between groups, even in high concentrations of serum. In the fixation test, as

seen in table 3, one must reach nearly to the titer limit of the serum, before obtaining evidence of group specificity.

THE EFFECT OF AGGLUTININ ABSORPTION

To test the effect on fixation of the absorption of agglutinins, the following experiment was made.

Serums representing the 3 groups was subjected to treatment with the respective antigens, until the serums, giving an original titer of 1 : 3,200, gave no agglutination at 1 : 10. To accomplish this, definite

TABLE 3

RESULTS OF TESTS ON 3 SERUM GROUPS WITH ALL ANTIGENS

Serum Group 1	Fixation		
	Antigen Group 1	Antigen Group 2	Antigen Group 3
0.025 c c.	+++	+++	+++
0.0125 c c.	+++	+++	++
0.00625 c c.	+++	++	Trace
0.003125 c c.	+++	Trace	0
0.0015625 c c.	++	0	0
Serum Group 2			
0.025 c c.	+++	+++	+++
0.0125 c c.	+++	+++	++
0.00625 c c.	++	+++	Trace
0.003125 c c.	Trace	+++	0
0.0015625 c c.	0	+++	0
Serum Group 3			
0.1 c c.	++	+++	+++
0.05 c c.	+	+++	+++
0.025 c c.	Trace	+	+++
0.0125 c c.	0	0	+++
0.00625 c c.	0	0	+++
0.003125 c c.	0	0	+++
0.0015625 c c.	0	0	++

amounts of culture were added to equal parts of serum, the whole incubated over night, and the supernatant fluid removed after centrifugalization. This was repeated, and after the second treatment with the bacteria the supernatant fluid was found to contain no agglutinins. Unextracted and normal serums were simultaneously carried through the same incubation periods, as a check on any possible alteration caused by heat. Fixation tests, using agglutinin-free serum, gave rather interesting, clear-cut results.

Cross fixations, that is, the use of the agglutinin-free serums with other than the homologous antigens, showed that all fixing bodies had been removed with the agglutinins. One might believe this fair evidence of the unity of antibodies, but when the difference in the rate of production and titer limits of these two reactions—fixation and agglutination—are considered, there seems to be a discrepancy (table 1).

SUMMARY

From many experiments it is evident that groups within the species B. diphtheriae cannot be as easily demonstrated by the fixation reaction as by agglutination.

Fixation, however, if carried to high dilutions, shows definite groups. In lower dilutions, fixation is not group specific.

The absorption of agglutinins from immune serum takes out practically all fixing bodies.

Since the rate of production of agglutinating and fixing bodies varies both in time and in degree, the results of absorption tests do not seem to be sufficient evidence on which to base any theory of the unity of antibodies.

A STUDY OF ANTIGENIC PROPERTIES OF BACTERIOPHAGE

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The phenomenon of bacteriophagy was first observed by Twort.[1] This author described a translucent change that took place in staphylococcus colonies inoculated on agar from vaccinia. These translucent areas transplanted to fresh agar sometimes gave a similar growth, but many times no growth occurred. A translucent or glassy colony subcultivated on agar along with a normal colony showed a predominance of the glassy or translucent colonies. When these colonies were diluted with water and passed through a porcelain filter, the filtrate when mixed with staphylococcus on an agar slant reproduced the glassy colonies.

D'Herelle [2] described a similar reaction for B. dysenteriae Shiga. He found that when a mixture of broth and feces from patients recovering from dysentery was incubated for several hours and then filtered, a clear sterile filtrate was obtained, possessing the property of clarifying a thick suspension of dysentery bacilli. If a trace of this cleared suspension was added to a second suspension of the bacilli, clarification again took place. This could be continued indefinitely. This phenomenon of microbic transmissible autolysis (Bordet) is usually referred to as "d'Herelle's phenomenon." D'Herelle considers this phenomenon to be brought about by an ultramicroscopic virus, which is a parasite of the bacteria. Lysis of the bacteria is a result of the growth of this ultramicroscopic parasite.

Bordet [3] thinks this lytic phenomenon, instead of being due to the agency of an invisible parasite, is in reality an autolysis, a process of autophagy. According to this author, microbic lysis may be regarded as the consequence of a nutritive vitiation of the bacterial metabolism, primarily induced by some external disturbing influence. Bordet conceives that this vitiation would cause the production of a lytic chemical principle, a very active substance, determining the lysis, and, moreover,

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[1] Lancet, 1915, 2, p. 1241.
[2] Compt. rend. Acad. d. sc., 1917, 165, p. 373.
[3] Compt. rend. Soc. de biol., 1921, 84, p. 280.

capable of communicating the same vitiation to new microbes belonging to the same species when brought into contact with them. A certain number of these microbes would not be immediately destroyed, and their multiplication would reproduce the lytic substance. Bordet has shown that leukocytic exudation under the same conditions will bring about this autolytic process in certain bacteria.

Kuttner [4] and Davison [5] have recently reviewed the literature on "d'Herelle's phenomenon." The various theories as the nature of this lytic principle are given in detail by these authors. We will restrict ourselves to a brief review of that part of the literature dealing with the antigenic properties of the bacteriophage.

Bordet and Ciuca [3] were the first to report the antilytic properties of serum following bacteriophage injection. This antibacteriophage serum neutralized the lytic action of the bacteriophage in the presence of a susceptible strain of bacteria.

D'Herelle,[6] in summarizing the properties possessed by antiserum produced by the injection of his bacteriophage, concludes that such antibacteriophage serum contains:

(1) Antibodies against the particular strain of bacteria used: agglutinins, precipitins and amboceptors.

(2) Antibodies against the bacteriophagous ultramicrobe: amboceptor and agglutinins.

(3) Antibodies against the specific lysins produced by the bacteriophagous ultramicrobe; antilysins.

The antibodies against the bacteria are due to the presence of bacterial proteins in the bacteriophage suspension injected. The antibodies against the bacteriophagous ultramicrobe are nonspecific. He thinks there is but one bacteriophage and that the amboceptor will fix complement no matter which strain of bacteriophage is used, anti-Shiga, antityphosus, antistaphylococcus, antiplague, etc. The antibodies against the lysins produced by the bacteriophage ultramicrobe are specific. This antilysin inhibits the lytic action on the bacteriophage. After several transplants from a mixture of antilysins and bacteriophage, the lytic activity will again appear. D'Herelle concludes that there is not a true neutralization, but only an inhibition. These results have been substantiated by Bail [7] and Watanabe.[8]

[4] Jour. Bacteriol., 1923, 8, p. 49.
[5] Abst. Bacteriol., 1922, 6, p. 159.
[6] Le Bacteriophage. Son role dans l'immunite, 1921.
[7] Wien. klin. Wchnschr., 1921, 34, p. 237.
[8] Ztschr. f. Immunitätsf., 1923, 37, p. 106.

D'Herelle found that bacteriophage increases the phagocytic activity of leukocytes; this he concludes is specific for the bacteriophagous ultramicrobe and is proportional to the strength of the bacteriophagic activity of the suspension used. He attributes this increase in the phagocytic power as due to the lysin content. When bacteriophage-resistant strains of bacteria were used, the opsonic index was less than 1.

Bruynoghe and Maisin [9] obtained nonspecific complement fixation with serum, prepared from staphylococcus and typhoid bacteriophage. Otto, Munter and Winkler [10] compared the intensity of the complement-fixation of antibacteriophage serum with the (1) bacteriophage, (2) emulsions of bacteria, (3) autolysates of living bacteria, and (4) autolysates of dead bacteria. The fixation was complete with the bacteriophage or lysins, but not so strongly positive with the remaining 3 substances; the autolysates of living bacteria gave a stronger fixation than the autolysates of dead bacteria. Antibacterial serum, prepared in the usual way, gave the same intensity of complement-fixation with bacteriophage, emulsions and autolysates. Prausnits [11] reports a bacteriophage that was resistant to antilytic serum.

D'Herelle's bacteriophage is prepared by adding a small quantity of an active bacteriophage to a 12-18 hour old suspension of a susceptible micro-organism in broth. After incubation for a variable length of time, complete lysis occurs with a disappearance of the cloudiness of the bacterial emulsion. The tube is as clear as uninoculated broth. The bacterial proteins liberated from the bacteria as a result of lysis are present in this tube, along with the bacteriophage. Antiserum produced by the injection of this bacteriophage contains antibodies against the bacteria used in the production of the bacteriophage. The antibacterial serum differs from the antibacteriophage serum in that the former does not contain antilytic properties.

One of us [12] recently attempted to roughly separate the bacteriophage from the bacterial proteins. A thin layer of a sterile 2% agar in distilled water was poured into Petri dishes; after cooling, a sterile piece of tissue paper the size of the dish was placed on top of the agar, and a thin layer of nutrient agar poured on top of this paper. The surface of this nutrient agar was seeded with a lysogenic strain of bacteria.

[9] Compt. rend. Soc. de biol., 1921, 85, p. 1122.
[10] Ztschr. f. Hyg. u. Infektionskrankh., 1922, 96, p. 11.
[11] Centralbl. f. Bakteriol., I, O., 1922, 89, p. 187.
[12] Arnold, L.: Jour. Lab. & Clin. Med., 1923, 8, p. 720.

Our usual method was to seed the surface with a culture of a susceptible micro-organism and add a drop or two of a bacteriophage suspension (the amount depending on the strength), smear well with a bent glass spreader and incubate 18 hours. The top nutrient agar layer with the adjacent tissue paper can be removed easily, leaving the bottom sterile layer of agar. This bottom layer is removed, divided into small particles by passing through a coffee sieve and extracted with distilled water between a layer of chloroform and toluol. Ten c c. of water were added to each agar layer. After several hours in the shaking machine, and after remaining over night in the icebox, the fluid was filtered off. This was extract 1. The agar was returned to the flask and the same amount of water added; the extraction procedure was again carried out; this fluid was extract 2. In this manner the extractions can be carried on as long as desired.

The top layer with the tissue paper is discarded. As has been shown,[12] the bottom layer contains more bacteriophage than the top layer. Active bacteriophage can be extracted up to the 10th or 12th extraction. Bacteriophage prepared by this method does not contain as much bacterial proteins as in the d'Herelle method.

Technic

The broth was made in the usual maner, with 1% Difco peptone and 0.5% Liebig meat extract. The medium had an end point of P_H 6.8 to 7.2. No sodium chloride was added. For solid medium, 2% washed agar was added. D'Herelle's bacteriophage was prepared in the usual way. The bottom layer agar extracted bacteriophage was prepared as outlined: the extract used will be noted in the following experiments.

Antiserum was prepared by intravenous or intracardial injections in rabbits with the bacteriophage suspensions at 2 to 4 day intervals; 8 to 12 injections were given, in some instances as high as 23 different injections. After the 8th or 10th injection in this manner, the intraperitoneal routes were used. The first dose was 1 c c.; this was increased by 1 c c. each time with subsequent injections up to 8 c c.; this amount was then used for all remaining injections.

In the agglutination and precipitation work, the tubes were incubated for one hour and placed in the icebox over night before the final reading was made.

In determining the opsonic index, human leukocytes were used. The citrated blood was washed 3 times with sterile normal salt solution. Instead of Wright's capillary pipets, we used small test tubes of uniform diameter. Five hundredths c c. of each ingredient was used; after mixing well, the tubes were placed in the incubator for 8 minutes at 37 C. After centrifuging at high speed for 2 minutes, smears were made from the sediments and stained with toluidine blue.

In the chemical study we used the micro-Kjeldahl method for total nitrogen. Folin and Wu's method[13] for nonprotein nitrogen, and Brown's[14] formal titration method.

[13] Jour. Biol. Chem., 1919, 38, p. 81.
[14] Jour. Bacteriol., 1923, 8, p. 245.

OPSONIC PROPERTIES OF BACTERIOPHAGE

D'Herelle has reported at length on the opsonic properties of bacteriophage. Table 1 gives in condensed form the results of our experiment. The opsonic index decreased as the activity of the bacteriophage decreased. Autolysates of typhoid bacilli do not exert any influence on the opsonic index when substituted in the experiment instead of bacteriophage. The antibacterial serum (1 : 100,000 agglutination titer) exerted a greater effect on the opsonic index than the antibacteriophage serum. When bacteriophage was added to either of these serums the opsonic index increased in proportion to the opsonic activity produced by the two separately. We conclude that the opsonins in the antibacteriophage serum are produced by the bacterial proteins and not by the bacteriophage itself. To

TABLE 1

EFFECT OF BACTERIOPHAGE ON PHAGOCYTOSIS

	Phagocytic Index	Opsonic Index
D'Herelle's typhoid bacteriophage	2.8	9.3
Arnold's typhoid bacteriophage, 1 extr.	2.6	8.6
Arnold's typhoid bacteriophage, 4 extr.	1.8	6.0
Arnold's typhoid bacteriophage, 8 extr.	1.1	3.6
D'Herelle's staphylococcus bacteriophage	0.4	1.3
Typhoid antibacteriophage serum	5.8	19.3
Typhoid antibacterial serum	15.2	50.6
Arnold's typhoid bacteriophage, 1 extr., and typhoid antibacteriophage serum	8.3	27.6
Arnold's typhoid bacteriophage, 1 extr., and typhoid antibacterial serum	17.0	56.6
Control	0.3	1.0

substantiate this, we found that if the antibodies are absorbed from the antibacteriophage serum by repeated saturation with typhoid bacilli, this serum, although antilytic, does not influence the opsonic index. The staphylococcus bacteriophage did not exert any influence on the opsonic index of typhoid bacilli.

ANTILYSINS

Six rabbits were immunized with d'Herelle's typhoid bacteriophage, 9 rabbits were immunized with bottom layer agar typhoid bacteriophage; 3 with the 1st extract, 3 with the 4th extract and 3 with the 8th extract. One rabbit received 23 injections of the bottom layer agar bacteriophage; after the 21st injection the serum became weaker in its agglutinin titer. The toxicity of these bacteriophage suspensions on injections was in the following order: d'Herelle, 1st extract, 4th extract and 8th extract. Six rabbits were immunized with staphylococcus bacteriophage, 3 with d'Herelle's and 3 with bottom layer agar 1st extract. The antilytic activity of the serums was tested in the following way: Decreasing amounts of antilytic serum were placed on the surface of sterile agar plates (3, 2, 1 drops, etc.) that were seeded with a uniform amount of a susceptible bacteria and 2 drops of an active bacteriophage; these were well distributed over the surface with a bent glass spreader. Antibacterial and normal serums were substituted in place of antilytic serum as controls. Proper controls for sterility of each bacteriophage and serum were used. Antitryptic serum was prepared and used as controls. All plates were incubated 18-24 hours. Normal, antibacterial and antitryptic serums did not exert any antilytic activity.

The antibacteriophagic serums showed antilytic properties. Antibacteriophagic staphylococcus serum does not interfere with lysis when added to plates seeded with typhoid bacilli and typhoid bacteriophage. Antibacteriophage typhoid serum has no effect on lysis produced by the staphylococcus and its bacteriophage when all 3 are seeded in proper proportions on agar plates. The specificity of lysins and antilysins was substantiated by this experiment.

AGGLUTINATION

The results obtained in the agglutination experiments with the 15 antiserums are as follows: 3 d'Herelle's typhoid antibacteriophage serums, 1:5120; 2 serums 1:2560 and the 6th serum 1:10240; three Arnold's typhoid antibacteriophage serums, 1st extract, 1:640; three Arnold's typhoid antibacteriophage serums, 4th extract, 1:320, and 3 for the same antibacteriophage serums, 8th extract, 1:80.

It will be noted that the titer of the d'Herelle's bacteriophage antiserums is as high as is usually obtained by using typhoid bacilli as the antigenic source.

TABLE 2

PRECIPITATION OF BACTERIOPHAGE WITH ANTIBACTERIAL AND ANTILYTIC SERUM

Typhoid Bacteriophage	Immune Serum Prepared with:				
	Typhoid Bacteria, Agglutination Titer 1:100,000	Typhoid Bacteria, Agglutination Titer 1:10,000	Typhoid Bacteriophage (D'Herelle's Method) Agglutination Titer 1:5,000	Typhoid Bacteriophage (Arnold's Method) Agglutination Titer 1:640	Staphylococcus Bacteriophage (D'Herelle's Method) Agglutination Titer 1:100
	Results				
D'Herelle's method, not passed through Berkefeld filter	1:5120	1:320	1:160	0	0
D'Herelle's method, passed through Berkefeld filter	1:640	1:40	1:20	0	0
Arnold's method, 1st extract, not passed through Berkefeld filter	1:1280	1:80	1:40	0	0
Arnold's method, passed through Berkefeld filter	1:320	1:20	0	0	0
Arnold's method, 2d extract	1:160	0	0	0	0
Arnold's method, 3d extract	1:80	0	0	0	0
Arnold's method, 4th extract	1:40	0	0	0	0
Arnold's method, 5th, 6th, 7th, 8th extracts	0	0	0	0	0

PRECIPITATION

Table 2 gives the results of the experiments dealing with the precipitins in the bacteriophage suspensions, prepared by the two methods, against antibacterial and antilytic serums. The differences between the bacteriophage suspensions passed through Berkefeld filters and the same suspension not passed through this filter are to be expected. There are many bacteria present in the latter. The growth of resistant strains that occurs so often after complete clearing indicates that the culture was not sterile. The larger bacterial particles are also held back by this filter.

The bottom layer agar extraction method yielded a bacteriophage with a lower bacterial protein content. This is clearly shown by glancing at the table. With an antibacterial serum, titer 1 : 100,000, the 4th unfiltered extract was positive only up to 1 : 40, and the 5th extract was negative.

The antibacteriophage serum made with d'Herelle's bacteriophage gave a titer of 1 : 10,000 with the homologous strain of bacteria. The bottom layer agar bacteriophage, 1st extract, only gave a titer of 1 : 640. The precipitation tests with these antiserums and various bacteriophages are in keeping with the titer of the respective antiserums compared with their agglutination titers. Staphylococcus bacteriophage antiserum did not give precipitation with the bacteriophages from typhoid bacilli.

The d'Herelle typhoid antibacteriophage serum with an agglutination titer of 1 : 5000 was repeatedly saturated with typhoid bacilli in concentrated form; this was repeated until all the agglutinins were absorbed. This serum was then found to be free of precipitin when tested against d'Herelle's bacteriophage. When we removed the bacterial protein antibodies, we could no longer get a precipitation with a bacteriophage.

TABLE 3

COMPLEMENT FIXATION WITH BACTERIOPHAGE

Total volume 2.5 c c.; amount of antigen, immune serum, complement and amboceptor as found by titration: each time at 37 C. for 1 hour in incubator; first reading after the second incubation, second reading the next morning		Immune Serum Prepared with							
		Typhoid						Staphylococcus	
		Bacteria	Bacteriophage					Bacteria	Bacteriophage Prepared with D'Herelle's Method
			Prepared with D'Herelles Method		Prepared with Arnold's Method				
				Agglutinins Absorbed	1st Extract	4th Extract	8th Extract		
Antigens: Staphylococcus Typhoid	Bacteria	++++	++++	—	+++	++++	+++	—	—
	Bacteriophage (D'Herelle)	++++	++++	—	++++	++++	+++	—	—
	Bacteriophage (Arnold) 1st extract	++++	++++	—	++++	++++	+++	—	—
	4th extract	++++	++++	—	++++	++++	+++	—	—
	8th extract	++++	++++	—	++++	++++	+++	—	—
	Bacteria	—	—	—	—	—	—	++++	++++
	Bacteriophage (D'Herelle)	—	—	—	—	—	—	++++	++++

COMPLEMENT FIXATION

There are at present a number of contradictory reports in the literature concerning the complement fixation of the bacteriophage. The complicated procedure used in this reaction, as compared to the simpler precipitation and agglutination reactions, probably partly explains the varied results reported.

In complement fixation of any kind and particularly when unknown antigenic substances are used, special attention must be given to the following points: (1) total volume, (2) careful titration of antigen, antiserum, complement and

amboceptor, (3) concentration of the red blood cells, (4) kind and duration of incubation, and (5) method of reading the results.

The titration of a great number of different bacteriophages and antilytic serums proved to us the necessity of titrating carefully the bacteriophage and the antilytic serum in each instance before proceeding with the complement fixation. The neglect of this simple procedure explains many of the contradictory and conflicting reports that have appeared. Antibacteriophage serum, prepared with both methods, gave specific fixation with the respective bacteriophages and bacteria. Antibacteriophage serum gave fixation with the respective bacteriophage. Nonspecific complement fixation as reported by some authors, including d'Herelle's, could not be obtained.

An undiluted typhoid antibacteriophage serum, which showed on the plates a definite antilytic activity and agglutinated typhoid bacilli in dilutions of 1 : 10,000, was saturated with large amounts of typhoid bacilli in concentrated form and incubated for 1 hour, then placed in the icebox over night. This serum was centrifuged until clear, removed from the sediment and the process repeated until all the agglutinins were absorbed. The antilytic activity of this serum had not been interfered with by this procedure. The fixation with this serum and the respective bacteria, or any kind of bacteriophage, could not be obtained (table 3). The results of this experiment would indicate that fixation in the first instance was due to specific bacterial proteins, and that probably the bacteriophage does not produce amboceptors.

Lysis or Antolytic Ferment

The complete clearing of a suspension of young bacteria by the addition of a bacteriophage led us to examine this cleared suspension as to its nonprotein nitrogen content. We were able to find by serologic methods that such a cleared suspension contained specific proteins of the lysed bacteria. If only lysis

TABLE 4

Comparison Between Bacteriophage and Typhoid Suspensions

	Control Broth		Cleared Bacteriophage Typhoid Suspension		Control Typhoid Suspension	
	Total N	NPN	Total N	NPN	Total N	NPN
Table I	149.8	76.2	175.4	72.5	173.6	85.6
Table II	150.2	76.4	175.4	73.5	175.4	85.3
Table III	150.1	76.2	175.3	72.8	170.1	85.1
Table IV	151.0	76.3	175.4	72.7	172.5	84.2
Table V	149.7	76.2	175.2	73.2	170.0	85.3

occurred, the specific proteins would be present in this clear fluid; but if in addition to lysis, digestion had taken place, we would expect to find the nonprotein nitrogen increased as compared with a control bacterial culture of the same age.

Twelve-hour old agar slant cultures of B. typhosus were emulsified in 5 c c. of normal salt solution. Three c c. of this emulsion were added to 15 c c. of broth, and to one set of tubes 1.5 c c. of bacteriophage were added; the other set was used as control. These were incubated for 18 hours. From previous experiments we had determined that it took this length of time for complete clearing

to take place. Table 4 gives the results of a few experiments. The results do not show an increase in the nonprotein nitrogen of the cleared suspension of typhoid bacilli. The differences in the total and nonprotein nitrogens of the cleared and cloudy suspensions can be explained by the growth that took place in the tubes to which bacteriophage had not been added. These tubes showed heavy growths and were centrifuged for a few minutes to avoid pipetting clumps of bacilli and introducing a greater error. The formol titration was also negative. Similar experiments with staphylococcus gave comparable results.

We attempted to use Berkefeld candles for separating the bacteria from the suspensions and in this manner be able to follow hourly the nitrogen changes that might take place in the suspensions that were gradually clearing. We found that no two filters would give us chemically comparable filtrates, and the same filter cleaned and sterilized carefully gave filtrates that could not be compared with each other. At times the nitrogen contents were increased and again decreased.

DISCUSSION

We have mentioned the theories of d'Herelle and that of Bordet in reference to the nature of the bacteriophage phenomenon. The tormer conceives this phenomenon as brought about by a living virus, the latter as an autolytic process brought about by some external influence. On closer study these two theories do not differ so much as would seem apparent on the surface. D'Herelle thinks the virus of bacteriophage secretes a substance, a lysin, that causes the dissolution of the bacteria. He says the bacteriophage is nonspecific, but the lysin secreted is specific for the susceptible bacterium. Bordet thinks the lysis is brought about by a nutritive vitiation, which is a product of the bacterium itself due to some external influence. The two differ as to the source of the lysin; both agree that the lysin or the ferment-like substance that actually causes the dissolution of the bacteria is specific.

Bacterial proteins have been a component part of all of the bacteriophagic solutions used for the production of antilytic or antibacteriophagic serum by all the workers in this field. Although we have been unable to obtain an active bacteriophage absolutely free from all traces of bacterial proteins, we have been able to study, from an antigenic standpoint, bacteriophage in medium containing varying amounts of bacterial proteins. In some instances these proteins were only detectable by serologic methods after from 15 to 20 injections into rabbits. Such bacteriophages exerted a definite lytic action on susceptible strains of bacteria.

We have been unable to show by nonprotein nitrogen determinations and formo titrations that there was a proteolysis accompanying the

bacteriolysis that takes place when a heavy emulsion of bacteria are cleared by the action of a bacteriophage.

We think the increase in the phagocytic activity, caused by the addition of bacteriophage to the washed white blood cells and susceptible bacteria, was due to the action of the bacteriophage on the bacteria. The opsonic index was increased in proportion to the activity of the bacteriophage. The increase in the opsonic index found on the addition of both antibacterial and antibacteriophage serum was roughly a summation of the respective components when tried separately.

After absorption of the bacterial protein antibodies from bacteriophagic serum, by repeated saturation with the bacteria used in the production of the bacteriophage, we were unable to obtain either a precipitation, a change in the opsonic index or a complement-fixation with this antiserum against the bacteriophage. But this antiserum still possessed its antilytic properties, seemingly unimpaired as a result of the absorption of the protein antibodies of bacterial origin.

Antilysins are specific, inhibiting only the lysins of the homologous strain used in the antiserum production. The antilysins were the only antibodies of bacteriophage origin we could find in the antilytic or antibacteriophagic serum. We ran a parallel experiment with trypsin, producing antitryptic serum by the same method as we used in our antilytic serum work. The same results were obtained in both instances; that is, only inhibiting antibodies against the bacteriophagic or lytic activity with the one and tryptic inhibition in the other.

We have been unable to demonstrate by any method the nonspecific bacteriophagic antiody described by d'Herelle. We have only been able to demonstrate the specific antilysins in the antibacteriophagic serum.

SUMMARY

Bacteriophage increases the phagocytic power of leukocytes and is in direct proportion to the potency of the bacteriophage.

Antilysins are the antibodies of the bacteriophage, and their action is specific.

Antibacteriophage serum prepared by d'Herelle's method gives a higher agglutination titer than when prepared by the bottom layer agar method.

When the agglutinins are absorbed from antibacteriophage serum, this serum no longer gives precipitation or complement fixation with

the bacteriophage and does not exert any influence on the phagocytic activity of leukocytes. The antilytic properties of such serums are not decreased.

A nonspecific precipitation, complement-fixation or increase in phagocytic power could not be obtained.

The bacteriophage reacts antigenically like a ferment.

STREPTOCOCCI IN RELATION TO ETIOLOGY OF EPIDEMIC ENCEPHALITIS

EXPERIMENTAL RESULTS IN EIGHTY-ONE CASES

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Impressed by the close similarity between certain cases of encephalitis and poliomyelitis, and because of certain results obtained in the latter disease and the extremely specific effects of streptococci in other diseases, I decided to study the etiology of epidemic encephalitis not only from the standpoint of a filtrable virus, the usual basis of study, but from that of the possible relationship of bacteria that might be found in portals of entry, and in the brain and cord.[1] Details of results in one case only have been reported, in which a rather peculiar streptococcus was isolated from the brain and cord, and also from portals of entry, as in epidemic hiccup,[2] a closely related condition, and with it symptoms and lesions were reproduced in animals.[3] In epidemic hiccup, spasms of the diaphragm were produced with the dead streptococci and with filtrates of young, actively growing cultures at the time the living culture, even after many rapidly made subcultures, induced this symptom.[4]

I shall report the results obtained during the last 4 years in a study of 81 cases of encephalitis.

METHOD OF STUDY

It seemed to me that whatever the causative organism of encephalitis might be, it undoubtedly had marked neurotropic properties, and that its affinity for the central nervous system might separate it from the other bacteria normally present in portals of entry. Intravenous injection of mass cultures was first tried, but the central nervous system is so thoroughly protected against bacterial invasion that localization was not sufficiently constant for routine work. Intracerebral inoculation of small doses was successful in causing a separation of the neuro-

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[1] Rosenow, E. C., and Jackson, G. H., Jr.: Jour. Infect. Dis., 1923, 32, p. 144.
[2] Jour. Am. Med. Assn., 1922, 79, p. 443.
[3] Jour. Infect. Dis., 1923, 32, p. 41.
[4] Ibid., 1923, 32, p. 72.

tropic organism from other bacteria obtained from the throat, by providing the former with a good medium for growth and the latter with conditions leading to destruction.

Material for cultures and for the injection of animals was obtained from the nasopharynx with a wire swab bent to the proper angle, without touching the tongue; from pus expressed from tonsils and aspirated from the depths of pyorrheal pockets, or from the apexes of extracted pulpless teeth in all cases, and also from the brain and medulla in fatal cases. The material from the portals of entry, suspended in 2 c c. of a sodium chloride solution, was used for cultures and animal inoculations. The cultures were made by spreading a small amount of the suspension on blood agar, and by inoculating glucose-brain broth with the swab, after washing in the 2 c c. of sodium chloride solution, and were incubated in a moist atmosphere at from 33 to 35 C. The blood agar contained 1 part of defibrinated horse blood to 19 parts of plain agar. The glucose-brain broth was prepared from meat infusion by adding 0.2% glucose, bringing the reaction to a hydrogen-ion concentration of 6.8, placing it in columns 10 cm. tall, in test tubes 20 by 1.5 cm., adding about 3 gm. of fresh calf, sheep, or beef brain, and autoclaving in the usual manner.

Various other procedures were used in making cultures from filtrates, and from the brain and cord of fatal cases. Immediately before inoculating the glucose-brain broth, it was heated in an Arnold sterilizer to drive off oxygen; ascitic fluid was added to some of the tubes, and others were layered with melted petrolatum. Tall tubes of meat infusion similar to those used for the cultivation of anaerobes were treated in the same way. Noguchi's medium was also inoculated.

The efficacy of the filters of the Mandler type was controlled with B. prodigiosus. The fluid was drawn through the filters by a water suction pump. Five % emulsions in sodium chloride solution or broth were prepared by grinding in a mortar with sand, pieces of brain, pons and medulla of fatal cases, about 1 c c. in volume, for culture, animal inoculation, and filtration. At first, rabbits were injected intracerebrally (under light ether anesthesia) with the sodium chloride solution suspension of the material from the nasopharynx or with the cultures therefrom, in series of 4, with 0.2 c c. and 0.1 c c., respectively, of the undiluted suspension or culture, and with the same amounts of suspension and culture diluted 1 : 100 with sodium chloride solution. It soon became apparent that injections of 0.2 and 0.1 c c., of the suspension, and these amounts of the culture

diluted 1 : 10, produced symptoms in a sufficiently short time to permit isolation of the apparent causative organism in pure, or nearly pure, form. If symptoms did not develop following direct injection of the sodium chloride solution suspension, or if extraneous organisms caused death from meningitis, the primary culture in glucose-brain broth, or more often after one or more rapidly made subcultures, or growths in this medium from suspected colonies on blood agar, was also injected. A somewhat peculiar green-producing streptococcus was found to be the common cause of death in the animals so injected. The effect of pure cultures after many transfers (as high as 73), through several animal passages, often in high dilution (as high as 1 : 1,000,000), was tested. In order to hold the specific symptom-producing power, transfers were made into warm broth, from 4 to 8 times every 24 hours, from the growth in the bottom of each preceding culture. Several platings on blood agar and even daily transfers in glucose-brain broth were not sufficient to maintain specific symptom-producing power. At first inoculations were made intracerebrally under ether anesthesia in the silent area of the right frontal lobe, and later subdurally near the median line far forward over the brain, or in the parietal region, by the methods of Loewe and Strauss, and Thalhimer, and in some instances in the cisterna magna. The dose for intracerebral inoculations of spinal fluid filtrates and brain emulsions ranged from 1 to 2.5 c c., and for intravenous injections from 3 to 5 c c. for medium sized rabbits. Localization after intravenous injection, as well as after packing the nose with gauze soaked in the culture, was often facilitated by simultaneous intracerebral injection of sterile horse serum, broth, or sodium chloride solution. In some instances mice were injected intraperitoneally with from 1 to 1.5 c c. of the suspension in sodium chloride solution of the material from the nasopharynx, or of the culture of the streptococcus. Loss of fluid after withdrawal of the needle was prevented by immediate firm pressure with sterile gauze. The procedures appeared harmless and were readily accomplished. The rabbits were given oats, hay, greens and water. They were observed at stated intervals morning, noon and night, and in special instances during the greater part of each day and night, and the symptoms were recorded as they developed.

Necropsies were made as soon after death as possible. The cultures of the brain and the spinal fluid were made by opening the skull in a sterile manner, turning back the vertex, pipetting the substance of the brain and the ventricular and spinal fluids, and inoculating blood-agar

plates and glucose-brain broth. Cultures of the blood from the heart, from 0.2 to 0.5 c c., were always made in the same mediums. The brain, medulla, and cervical cord were examined and placed in 10% formalin for microscopic study.

Blocks for microscopic study were taken routinely from the cerebral hemisphere, the pons, the medulla, including the posterior part of the cerebellum, and the cervical cord. In special instances, sections were made from numerous levels of the brain and cord. The sections were stained for lesions with hematoxylin and eosin, and for bacteria by the Gram method, except that methyl or crystal violet instead of gentian violet was used. The sections were decolorized to a pale blue, so that the nuclei of cells were pale blue and the bacteria dark purple, or almost black in transmitted light.

RESULTS IN THE LETHARGIC GROUP

In 13 of the 81 cases studied, lethargy was a prominent symptom, either before or at the time of my study. The ages of the patients ranged from 7 to 53 years. Eight were males, and 5 females. The duration of symptoms ranged from 9 days to 3 years; in one, it was 9 days; in one, 10 days; in four, 1 month; in two, 2 months; in three, 4 months; in one, 2 years, and in one, 3 years. Besides lethargy, the most common symptoms were weakness or paralysis, with or without tremor, twitchings in one or more groups of muscles, and nystagmus. The incidence of symptoms in the animals is given in table 1. Of the 159 rabbits injected with material known to contain the streptococcus, 39 became lethargic, in sharp contrast to the incidence of lethargy following injection of the streptococcus from other forms of encephalitis. Moreover, this symptom did not develop in any of the 106 rabbits injected in the same way with material from 77 normal persons. Paralysis developed in 53 of the 159 animals injected, tremor in 52, spasm of muscles in 17, hyperpnea in 37, ataxia in 29, and nystagmus in 14.

Illustrative Cases

Case 1.—A woman, aged 41, a patient of Dr. A. S. Hamilton, Minneapolis, on Feb. 10, 1920, coincidental with a social affair at her home, developed restlessness and insomnia, followed in 4 days by an abnormal sense of fatigue, drowsiness, and sudden severe pain running from the left eye to the left ear. The pain soon disappeared, and she slept almost constantly for 2 weeks, when she again became wakeful, moderately restless, slow in response to various stimuli, and her eyelids drooped.

The temperature ranged from 97 to 99.2, and the pulse from 80 to 120. There were ptosis of the eyelids, slightly greater on the right, marked slowness

TABLE 1

INCIDENCE OF SYMPTOMS AND OF GREEN-PRODUCING STREPTOCOCCI, AND MORTALITY IN ANIMALS FOLLOWING INTRACEREBRAL INJECTION OF MATERIAL CONTAINING THE STREPTOCOCCUS

Source	Number of Strains	Rabbits		Mortality (percent)	Spasms of Muscles			Abnormal Movements and Posture							Restlessness	Hyperpnea	Convulsions	Paralysis	Lethargy	Animals Cultured	Green-producing Streptococci in	
		Injected	Died		Diaphragm	Abdominal	Other	Nystagmus	Rhythmic Movements of Head	Tic-Like Movements of Head	Ataxia	Turning of Head	Retraction of Head	Tremor							Brain	Blood
Lethargic encephalitis.	13	159	..	..	5	7	17	14	0	1	29	7	8	**52**	15	37	1	**53**	**39**			
Parkinsonian encephalitis.	15	89	..	..	1	5	12	7	3	0	**35**	12	6	**44**	13	17	0	**25**	5			
Myoclonic encephalitis.	17	77	..	..	**6**	**10**	**35**	6	5	3	27	7	17	**42**	10	24	4	19	5			
Meningitic encephalitis.	5	58	..	..	**12**	**15**	**27**	2	0	0	17	3	4	**33**	2	8	1	**32**	8			
Unclassified encephalitis.	34	136	..	..	1	3	24	11	3	1	50	10	7	49	17	39	1	40	10			
Total.....	81	519	384	74	25	40	115	40	11	5	158	39	42	220	57	125	7	169	67	276	200	89
Encephalitis contacts.....	11	30	12	40	0	0	4	3	0	1	14	10	0	5	0	7	0	4	2	29	8	2
Normal controls 1922 and 1923.....	77	106	24	23	0	0	2	7	1	2	17	19	2	4	2	10	0	8	0	97	24	7

of the movements of the eyeball, but no diplopia, circular and equal pupils, reacting slowly to light and accommodation, intermittent nystagmus, moderate rigidity of the neck, normal superficial and deep reflexes, and weakness of the muscles of the extremities, but no localized paralysis. The urine was normal, the hemoglobin 76, the erythrocytes 4,200,000, and the leukocytes 8,000. Spinal fluid and blood Wassermann reactions were negative; the spinal fluid was clear, under normal pressure, contained 6 cells, and gave no globulin response. During the first month at the hospital, the patient was very weak, and responded only to insistent requests. She ate little, and although apparently drowsy, did not sleep well. By May 27, improvement was marked. Ptosis had wholly disappeared; she was able to sit up in a chair, and take a few steps, but still slept poorly, and complained throughout the course of her illness of spells of difficult breathing.

Experiments.—The effect of the streptococcus from the nasopharynx of this patient 4 months after the onset of symptoms, when she was recovering, was studied through 6 successive animal passages. The strain was only moderately virulent. The one used for animal passage was mainly from rabbits that lived some time after injection. Little increase in virulence was noted. Of the 37 injected, 20 died. Nine developed tremor of the masseters and other muscles, 7 ataxia, 7 weakness of the extremities, 5 meningitis, 6 nystagmus, and 4 lethargy.

Rabbit 1882 was injected intracerebrally on May 30, 1920, with 0.5 c c. of the emulsion of the brain of a rabbit that died from encephalitis following injection of this strain after 4 animal passages, in the second subculture. Until June 1, 8:30 a. m., the animal appeared well. At 5 p. m., it was excitable, and had developed marked tremor of the muscles of the neck and fore extremities, slight unsteadiness of the head, and ataxia. June 2, 8:30 a. m., the condition was worse; weakness in the hind extremities had developed. The symptoms then gradually improved, until by June 16 the tremor and ataxia had disappeared, but the right hind extremity was weak, and the knee jerks were markedly increased, slight tapping on the patellar tendon causing clonus. The following day, the animal sat quietly. There was undoubted delay in response to stimuli, and it was definitely ataxic and weak in the hind extremities. On June 18, 3 p. m., the animal was found sitting quietly with eyes partly closed; respirations were slow. It responded slowly to stimuli, and hopped slowly and with difficulty, tending constantly to fall to the right. The knee jerks were normal. At 5 p. m., it was found apparently fast asleep, nodding its head repeatedly. Responses to stimuli were delayed. On June 19, 7 a. m., the lethargy was still more pronounced. When crumbs of bread were placed in its mouth, it chewed slowly for from 3 to 6 mastications, grinding its teeth audibly and violently, when it again appeared to fall asleep, forgetting to swallow. After vigorous prodding, it maintained its balance with difficulty, tending this time to fall to the left. At 8:30 a. m., the condition was much the same. The tonus of the muscles of the extremities was increased; slight stimulation repeatedly induced fine tremor and twitching of the muscles of the neck, the jaw, and the hind extremities. At 3 p. m., the respirations were rapid and irregular; the animal was weaker, and continued to sleep, responding only to severe prodding. On June 20, 6:30 a. m., it was found dead, apparently in the same position as when last observed.

Necropsy revealed a clear cerebrospinal fluid, moderate congestion of the vessels of the meninges, moderate softening of the brain and the cord, and a brownish area of infiltration at the point of injection in the right frontal lobe, approximately 5 mm. in diameter. There were no gross lesions in the brain, cord, or other organs. Cultures from the blood and spinal fluid remained sterile;

those from the brain yielded a pure culture of the streptococcus. Sections of the various levels of the brain, medulla, and cord revealed a widespread perivascular round-cell infiltration, most marked near the localized area of leukocytic infiltration in the right frontal lobe, in the corpus striatum, and in the basal ganglions (fig. 1), but also present in the subcortical region of the brain in the opposite hemisphere, and in the medulla and the spinal cord. The emulsion of the brain of this animal produced a similar type of encephalitis in two rabbits injected.

Comment.—This case is interesting because of the classic clinical picture, the isolation of the encephalitis coccus with which characteristic symptoms and lesions were produced in animals four months

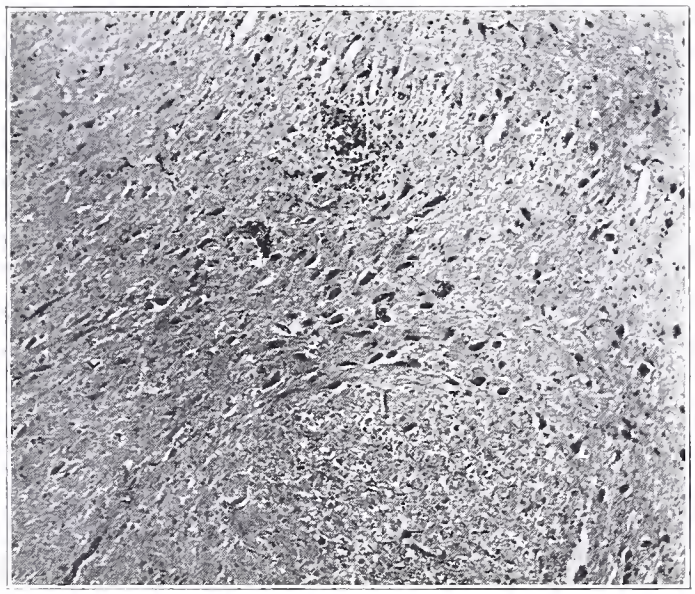

Fig. 1.—Localized and perivascular round-cell infiltration in brain stem of rabbit 1882. Hematoxylin and eosin (× 60).

after onset of the symptoms when the patient was recovering, and because the streptococci did not change perceptibly in cultural characteristics, localizing power, or agglutinating properties when passed slowly through a series of animals.

CASE 2.—A woman, aged 69, a patient of Dr. S. R. Maxeiner of Minneapolis, developed symptoms of acute encephalitis from no apparent cause. The attack began with headache, malaise, and diplopia. Extreme weakness and severe headache forced the patient to remain in bed on the third day. She then became increasingly somnolent, intermittent hiccup developed, and except for short lucid intervals she was completely unconscious after the fifth day.

The temperature was 102 F., the systolic blood pressure 175, and the leukocytes were 18,300. The tonus of the muscles of the extremities was increased; there was doubtful rigidity of the neck, and the eye grounds were normal.

The spinal fluid was clear, apparently under normal pressure, contained 10 cells, and the globulin reaction was weakly positive. The patient was better for 48 hours after the first injection of my encephalitis serum, but the improvement was temporary; subsequent injections were without effect, and she died without convulsions on the 10th day of illness.

Necropsy by Drs. McCartney and McKinley disclosed beginning bronchopneumonia. Microscopic sections showed localized hemorrhages and a small number of mononuclear cells, probably lymphocytes, surrounding small vessels in the medulla, pons, and basal nuclei, in the pia in sulci, and in the pons, in one area, typical cuffing surrounding a good sized blood vessel.

Experiments.—Cultures from the nasopharynx during life and from the emulsion of the pons, and the filtrate thereof, yielded identical streptococci which had marked and peculiar neurotropic power and were agglutinated specifically by the encephalitis and poliomyelitis serums.

One-tenth c c. of the suspension in sodium chloride solution of the throat swab was injected undiluted into 1 rabbit, diluted 1:50 into 2, and 1:500 into 1. The first 3 died within 48 hours with stormy symptoms, and the 4th in 8 days, from lethargic encephalitis. The streptococcus was isolated from the brain in all. The emulsion of the brain of the last rabbit was injected into 2 rabbits; one remained free from symptoms, the other developed lethargic encephalitis and died on the 6th day. Cultures of the brain yielded a few colonies of streptococci. The emulsion of this brain was without effect in 2 rabbits injected; both were anesthetized in 8 days, and no microscopic lesions were found. The cultures were sterile, and the strain was lost.

Four rabbits were injected subdurally with the streptococcus from the throat in the 15th rapidly made subculture in doses ranging from 0.2 c c. of the undiluted culture to 1:1,000 dilution. All but one developed symptoms; all had characteristic lesions in the brain, and the streptococcus was recovered from 3.

The streptococcus isolated from the filtrate of the emulsion of the pons in the 15th rapidly made subculture was injected subdurally into 3 rabbits, in doses of 0.2 c c. of a 1:10, 1:100, and 1:1,000 dilution of the 12th rapidly made subculture. The 2 injected with the high dilutions remained well; the other developed lethargic symptoms, died in 6 days, and had typical lesions. The streptococcus was recovered in small numbers from the brain, but the emulsion of the brain was without effect in 2 rabbits injected. They were anesthetized 8 days after injection; the cultures were negative, and sections revealed no lesions.

The streptococcus from the filtrate of the emulsion of the pons in the 18th rapidly made subculture was injected intracerebrally in varying doses into 3 rabbits. All developed lethargic symptoms and characteristic lesions, and cultures yielded the streptococcus. The emulsion of the brain of one that died in 10 days was transferred to 2 rabbits. One died of intercurrent infection; the other became definitely slow and sleepy and died in 5 days. Sections of the brain revealed characteristic lesions, and the streptococcus was recovered in cultures. The brain emulsion of this rabbit was in turn injected into 2 rabbits, both of which remained well for 4 and 5 months, respectively, and the strain was lost.

The emulsion of the pons of the patient and the corresponding filtrate, from both of which the streptococcus was isolated, were each injected into 2 rabbits. All remained free from symptoms for 12 days, when they were anesthetized. Cultures were sterile and sections were free from lesions.

The streptococcus from the throat and the filtrate of the emulsion of the pons in the 15th rapidly made subculture were injected intravenously into 2 and 4 rabbits, respectively. Localization occurred in the brain in 3, one of which became tremulous, and 2 lethargic. Characteristic lesions were found in these 3, whereas lesions were not found in the 3 that remained free from symptoms.

Of the 28 rabbits injected intracerebrally in varying doses with material known to contain the streptococcus, including the suspension of the throat swab, the emulsion of the pons, and the filtrate of this, the corresponding cultures after many rapidly made subcultures and after 2 and 3 animal passages, 7 developed lethargy; 14, lethargy or other symptoms of encephalitis; 1, spasms of the diaphragm; 1, spasms of muscles; 6, hyperpnea; 7, paralysis, and 3, tremor. In 14, characteristic lesions were demonstrated in the brain.

Comment.—The points of interest in this case are the short duration of symptoms, the relatively slight yet characteristic microscopic findings in the brain, the demonstration of the streptococcus in the lesions in the brain of the patient, the isolation of the identical streptococcus from the throat, the emulsion of the pons, and the filtrate of this, and the relatively high incidence of lethargic symptoms and typical lesions following both subdural and intravenous injection. The absence of symptoms following the injection of highly diluted cultures and emulsions of the brain of positive rabbits indicates clearly why the pons emulsion of the patient and the filtrate thereof, both of which were proved to contain the streptococcus, failed to produce symptoms or lesions.

CASE 3.—A man, aged 41, came to the Clinic on Feb. 16, 1923. Two weeks before, he had had nausea and high fever for several days. During convalescence from this he had a severe frontal and occipital headache and insomnia, which lasted for about a week, when he developed diplopia, extreme restlessness and delusions, and became mentally confused.

The patient was extremely restless, although he appeared half asleep much of the time. He had diplopia, tremor of the eyeballs suggesting nystagmus, tremor of the extremities, myoclonic movements of the muscles of the extremities and abdomen, marked pyorrhea, and infected tonsils, and the nasopharynx was hyperemic and abnormally moist. The leukocyte count was 10,800; the urine was normal; the blood pressure was normal; the spinal fluid contained 15 lymphocytes; the Nonne reaction was positive; Kolmer reactions of the spinal fluid and blood were negative. The patient was given the encephalitis serum with apparent benefit, and recovered slowly but completely.

Experiments.—The sodium chloride solution suspension of pus aspirated from pyorrheal pockets and the washings from the nasopharyngeal swab were each injected subdurally into a rabbit and intraperitoneally into a mouse. The rabbit receiving the pus from the teeth, and both of the mice, remained well. The findings in the rabbit receiving 0.1 c c. of the washings from the nasopharyngeal swab developed symptoms as follows:

Rabbit 4319 was injected on Feb. 16, 1923. The morning of February 17, it appeared well, but by evening it had developed hyperesthesia, tremor, and

twitchings of the muscles of the fore extremities, which lasted for 4 or 5 days, and gradually disappeared. On Feb. 28, it appeared well and ate normally, but had lost in weight. For about a week, the right ear drooped. On March 10, it appeared well, but seemed abnormally sleepy. The lethargy increased gradually for 12 days. It slept most of the time, with eyes half closed, holding its head and ears forward in a characteristic attitude (fig. 2 *a*), and only severe prodding aroused it. Pulling its "whiskers," moderate tapping and shaking, and even turning it on its back, a position to which normal as well as most sick rabbits object strenuously, often failed to awaken it (fig. 2 *b*). When made to hop it did so slowly and stiffly a few times, and when given food it frequently forgot to chew and swallow before falling asleep. The tonus of the muscles of the extremities was markedly increased, and they tended to stay in abnormal positions (fig. 2 *b*).

A

B

Fig. 2.—Rabbit 4319 injected intracerebrally 21 days previously with the streptococcus from the nasopharynx of a patient with lethargic encephalitis (case 3). Marked lethargy (*A*), and rigidity of extremities (*B*).

The animal was anesthetized for necropsy on March 23, thirty-six days after injection. No lesions were found. Cultures and filtrates of the emulsion of the brain, and cultures of the blood were sterile. Areas of necrosis with little or no cellular infiltration, irregular staining of ganglion cells, moderate neurophagocytosis, slight perivascular round-cell infiltration (fig. 3), and complete plugging of a small artery by round cells were found in the region of the corpus striatum, localized areas of round-cell infiltration in the subcortical region, and a few gram-positive diplococci in the lesions. The filtrate of the emulsion of the brain was injected subdurally into 2 rabbits. Both remained well and free from lesions. The 3 rabbits injected subdurally with the spinal fluid of the patient remained well.

RESULTS IN THE PARKINSONIAN GROUP

The parkinsonian syndrome was well developed in 15 patients. Their ages ranged from 3½ to 54 years. Five were females, and 10 males. The duration of symptoms ranged from 6 weeks to 4½ years; in 2 it was six weeks, in 5 one year or less, in 3 two years, in 3 three years, and in 1 each, three and one-half and four and one-half years.

Seven had had recurring attacks of tonsillitis, and all had a history of either a febrile attack resembling influenza, or undoubted acute encephalitis preceding the onset of the parkinsonian symptoms. A catarrhal pharyngitis with hyperemia of the throat was present in all.

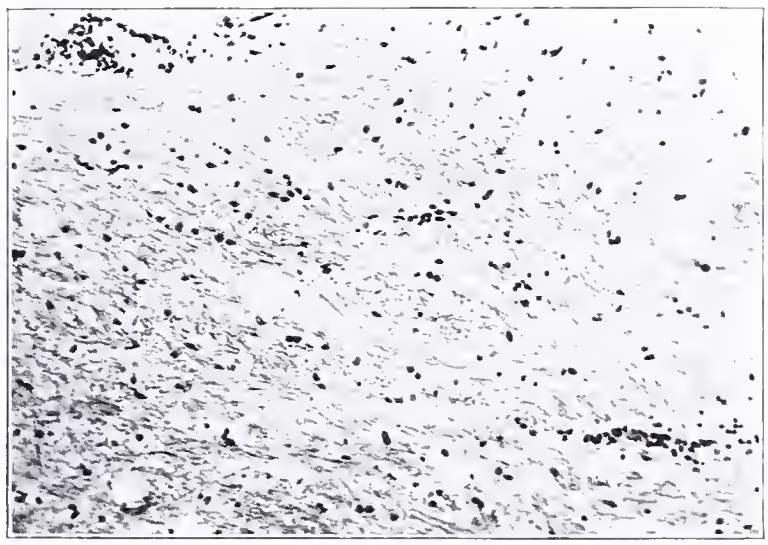

Fig. 3.—Localized and perivascular round-cell infiltration in the corpus striatum of rabbit shown in fig. 2. Hematoxylin and eosin (× 120).

Infected tonsils, from which pus was expressed, and devitalized teeth with areas of infection at the apexes, or with pyorrhea, were found in most instances. Abnormal slowness in all movements, characteristic tremors, difficulty in walking, and weakness of the extremities, in which the tremor was most pronounced, were the striking symptoms in this group of patients. Six had had clonic twitchings or spasms of muscles, and 5 lethargy. The incidence of symptoms in animals following intracerebral inoculation is summarized in table 1. Abnormal slowness, increased tonus of muscles, tremor, often severe, ataxia, and paralysis were the striking symptoms. The last three occurred in 44, 35, and 25

instances, respectively, of the 89 animals injected, spasms of muscles occurred in 12, and hyperpnea in 17, whereas only 5 became lethargic.

In one typical case, similar symptoms developed in 3 series of experiments performed with material obtained from the nasopharynx on 3 different occasions at intervals of 3 and 9 days. Of the 7 animals injected, 6 developed muscular rigidity, clonic spasms, and tremor of muscles, 2 marked salivation, and 4 abnormal slowness. The findings in this group are illustrated in cases 4 and 5.

Case 4.—A man, aged 21, came to the Clinic on April 10, 1920. He had been well until about 6 weeks before, when he complained of blurred and double vision. His physician made a diagnosis of lethargic encephalitis. The diplopia disappeared in 3 days, but during the succeeding 6 weeks the patient seemed disinterested in his surroundings and slept most of the time.

He was weak and stiff, especially in the arms, moved slowly, and responded slowly to questions. The abdominal reflexes were absent; the tendon reflexes of the upper extremities were slightly exaggerated; and sensations were normal. The muscles of the arms, particularly the right, were weak, and the muscles of the arms and of the jaw were distinctly rigid. He drooled saliva, and had difficulty in chewing his food, choking occasionally. The pupils were equal and reacted normally to light and accommodation; there was no diplopia; the eye grounds were normal; and vision was unimpaired. Response to questions was intelligent but delayed, and speech was slow. He was well oriented, but at no time did he carry on a conversation. He slept most of the time, but could be aroused without much difficulty. The temperature, pulse, and respiration were nomal; the systolic blood pressure was 145, diastolic 90, hemoglobin 80, and the leukocyte count 8,000; urinalysis revealed a faint trace of albumin; the spinal fluid contained 3 lymphocytes for each cubic millimeter; the Wassermann reaction was negative, and the Nonne reaction was faintly positive. After leaving the hospital, the patient improved markedly until the latter part of May, when twitching of the muscles of the left leg developed. This continued more or less constantly for 4 months, but in other respects his condition gradually improved. Early in September he had an attack of respiratory influenza, which was epidemic in his community at the time, associated with headache, fever, cough, and pains in the chest and the back. About 2 weeks after this attack the encephalitic condition grew worse. Movements and response to questions became slower; twitching of the muscles of the left leg continued; the weakness of the right arm increased; the mask-like expression became more marked; the tendency to sleep much of the day returned, and he was less interested in his surroundings.

Experiments.—The nasopharyngeal washings, and a suspension in sodium chloride solution of pus expressed from the tonsils, were cultivated, filtered and injected intracerebrally into rabbits on two occasions, April 10 and 13. A streptococcus which produced rather large, moist, flat, green-producing colonies on blood-agar was isolated, directly from the nasopharyngeal washings, from the salt solution suspension of the pus from the tonsils, and from the filtrates of these on both occasions, and also from the brain and spinal fluid of animals that succumbed to encephalitis following injections of these cultures. The effect on the freshly isolated strain of intracerebral inoculation of rabbits and monkeys was studied through 8 animal passages. The material for each sub-

sequent animal passage, with 2 exceptions, was selected from animals that lived from 5 to 9 days following inoculation. The virulence did not materially increase, and there was little change in the symptoms and type of the disease produced, or in the mortality rate. Of the 42 animals injected (39 rabbits and 3 monkeys), 17 (40%) died as a result of the injection. Clonic spasms of the muscles developed in 6; tremors of varying degree in 15; ataxia in 10; marked weakness or paralysis of one or more of the extremities in 16; lethargy in 2; and gross evidence of meningitis in 4.

Monkey 249 was injected on April 13 with the filtrate of the nasopharyngeal washings, 2 c c. into the right frontal lobe, and 3 c c. intraperitoneally. On April 16, the animal was excitable, and marked tremor, especially of the anterior part of the body, and slight incoordination were noted. On April 17, the tremor of the head was pronounced, and the animal was extremely excitable. The spinal fluid was clear. On April 19, the condition was about the same except that the knee jerks were exaggerated, and there was undoubted weakness of the muscles of the left side of the face and the fore extremities. On April 21, the condition was practically unchanged, and the animal was chloroformed.

Necopsy revealed moderate congestion of the vessels of the meninges, clear cerebrospinal fluid, and a number of small, brownish red areas resembling old hemorrhages, on the cut surface of the cerebrum. Blood-agar plate and glucose-brain broth cultures of the cerebrospinal fluid, of the emulsions of the pons and cerebrum, and of the blood, remained sterile. Cultures in meat infusion of the filtrate of the cerebrum and pons, and of the spinal fluid obtained on April 17, yielded a pure culture of the streptococcus. Sections of the brain and cord revealed localized round-cell and perivascular infiltration in the pia and the pons, and slight degeneration of the ganglion cells associated with moderate neurophagocytosis in the deeper layer of the cortex, the medulla, and the cord.

Rabbit 1792 was injected on April 22, at 4 p. m., with 0.2 c c. of the primary glucose-brain-broth culture in the second animal passage. At 9 p. m., there was general twitching of all muscles, varying in severity, at times bordering on convulsive attacks. On April 23, there was decided improvement, but weakness of the fore extremities and tremor extending over most of the body had developed. On April 24, the tremor of the head and of the muscles of the fore extremities was constant, and weakness of all extremities was marked. On April 26, there was noticeable improvement. The fore extremities were still weak, but the animal could hop. There was marked tremor and moderate rigidity of the muscles of the jaw. On April 27, the condition was about the same, and the animal was chloroformed. The ventricular and cerebrospinal fluids were increased in amount; the surface of the brain was wet, and the vessels congested, but there were no gross lesions at any point, including the site of inoculation. Cultures of the blood were sterile; those of the fluid from the ventricles and frontal sinuses yielded a pure culture of the streptococcus. Cultures from emulsions of the brain were sterile, the 2 rabbits injected with this filtrate remaining free from symptoms. Sections of the brain revealed localized round-cell infiltration around the wall of the 4th ventricle, slight infiltration around the vessels of the cerebrum, and marked degeneration of the ganglion cells in the medulla and the cord.

The strain in the 6th animal passage was grown in large amounts of glucose broth for the immunization of horses early in May. This growth was removed from the fresh broth culture by a continuous feed centrifuge, and the bacteria were placed in a dense suspension in glycerol (2 parts) and 25% sodium chloride solution (1 part). This suspension was kept on ice until August 17, when a

blood-agar plate culture was made. On Aug. 18, there were numerous typical colonies on the blood-agar plate, and it was decided to retest the infecting power in animals. A suspension in sodium chloride solution was made of a dilution approximately equal to that of the broth culture. This was diluted 50 times, and 2 rabbits were inoculated intracerebrally with 0.2 c c. each. Both developed encephalitis. One had marked tremor, ataxia, weakness, and salivation, and died 3 days later. The other had similar symptoms at first, and then developed rigidity of the muscles of the jaw and of the extremities, fine and coarse tremor and twitching of the left side of the face, and increasing lethargy, and died 1 week after inoculation. Cultures from the brain of both yielded a pure growth of the streptococcus injected, and sections revealed typical lesions. The strain in the 6th animal passage was also inoculated into tall tubes of meat infusion, incubated for 24 hours, and kept in the dark at room temperature until Aug. 4, 1921, when another series of experiments was performed, in which the infecting power of the strain was again tested. The effect of the filtrate of this old culture was tested by injecting intracerebrally amounts varying from 1.5 to 0.5 c c. into rabbits, and 1 c c. intraperitoneally into mice. The filtrate was found to be extremely toxic. Immediately after intracerebral injection, the animals developed marked tonic rigidity of all muscles, which continued for from 1 to 2 hours, when they relaxed, became weak, and respirations increased. The cultures from the brain of 7 rabbits that died the 1st or 2d day following injection remained sterile, whereas the characteristic streptococcus was isolated from the brain and the cord of one that died on the 5th day. The culture in glucose-brain broth from the old meat infusion culture was injected intracerebrally into 2 rabbits. Both developed a similar train of symptoms, illustrated in the protocol of rabbit 2638.

Rabbit 2638 was injected on May 4, 1921, with 0.1 c c. of the growth in glucose-brain broth from the old meat infusion culture. On May 5, it appeared well. On May 6, it sat quietly, with marked fine tremor of the head and the fore extremities. It appeared afraid to move, and when forced to do so, the tremor increased, and it became excited. On May 7, at 6:40 a. m., continuous peculiar movements of the head and the fore part of the body, resembling paralysis agitans, had developed; this became worse when the animal was forced to hop. It appeared quite well otherwise, licked its paws, and ate normally. On May 8, at 9 a. m., it appeared well and ate normally, but the tremor had become so extreme that the whole body shook when it hopped, and at times while it sat quietly. At 3:30 p. m., the tremor was less marked when the animal was quiet, but when it was prodded, and as it became quiet afterward, spells of severe shaking resembling the marked tremor of paralysis agitans developed. When forced to hop, weakness and decided rigidity of all extremities were noted. On May 9, the animal was found dead.

At necropsy, no gross lesions were found. Cultures from the emulsions of the brain yielded a pure growth of the streptococcus, whereas those from the filtrate of the emulsion of the brain and those from the blood remained sterile. Sections revealed widespread infiltration, chiefly by round cells, in localized areas and around blood vessels, most marked in the corpus striatum and subcortical region. The filtrate of the emulsion of the brain of this rabbit was injected into 2 rabbits, both of which remained free from symptoms. The primary culture from the emulsion of the brain also was injected into 2 rabbits. One remained well; the other became lethargic. The emulsion of the brain of this animal was injected into 2 rabbits. Both developed symptoms of encephalitis and died, one in 7 and one in 9 days. Both showed typical lesions of the brain on microscopic examination, and cultures yielded the streptococcus.

The toxicity of the old meat infusion culture was also manifested on intraperitoneal injection into mice. Shortly after inoculation, the mice developed marked tremor and twitching of the muscles of the extremities, tail, neck, and head, varying greatly in intensity from time to time. The attacks were precipitated repeatedly by slight stimulation. The tremor and twitching disappeared the day after injection, respirations were slow and shallow, and the animals appeared half asleep, responding slowly to prodding. They could be awakened readily, but would again fall fast asleep. The lethargy gradually became less, and recovery ensued.

An immune serum prepared by repeatedly injecting a horse with increasing doses of the suspension in glycerol agglutinated specifically in high dilution the organisms preserved in this suspension, and the streptococcus from the 1-year-old meat infusion culture, but did not agglutinate the same strain grown on blood-agar slants. The streptococcus was isolated by direct cultures from filtrates of

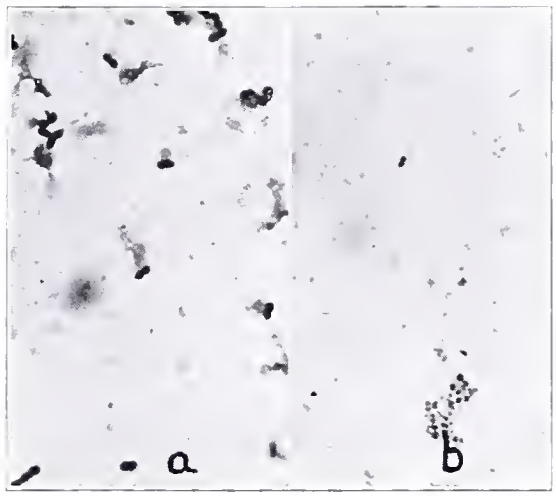

Fig. 4.—Smear preparations of strain from case 4. *A*, 24-hour culture in glucose-brain broth; note the large diplococci of uniform size. *B*, 10-day culture in ascites-tissue fluid; note the medium sized and the extremely small poorly stained diplococci. Gram stain (× 1000).

washings from the nasopharynx and from the brain of animals that developed symptoms of encephalitis following injection of the filtrates, from 2 filtrates of the old meat infusion culture, containing extremely small forms of the organism, and from 2 of 4 filtrates of emulsions of brain and cord of 4 positive rabbits in sodium chloride solution. The B. prodigiosus, which was used to test the efficacy of the filters, was not found in the filtrate in a single instance.

Comment.—The points of particular interest in this case are the changing character of the symptoms in the patient, the initial attack being typical lethargic encephalitis, with later development of myoclonic twitchings and marked parkinsonian syndrome; the reproduction of the symptoms and typical lesions in animals when the strain was first isolated in a series of animal passages; the preponderance of the

parkinsonian type of tremor in animals injected with the strain after preservation in latent life in meat infusion for one year; the extreme toxicity of the filtrate of the old meat infusion culture; the presence of the characteristic streptococcus in filtrates of nasopharyngeal washings and the old meat infusion culture; the marked difference in size of the organisms, depending on the medium (fig. 4 *a* and *b*), and the preservation of specificity of the strain when kept in latent life and in dense suspension in glycerol-salt solution, making the production of a therapeutic serum possible.

Case 5.—A man, aged 46, came to the Clinic on March 17, 1923, on account of a progressive slowness of gait, movements, and speech; pains in the left forearm and back part of the thigh; cramping of the muscles of the thigh and calf, which had come on gradually from no apparent cause, and trembling of the throat and tongue, which had developed 3 weeks before, soon after an attack of influenza.

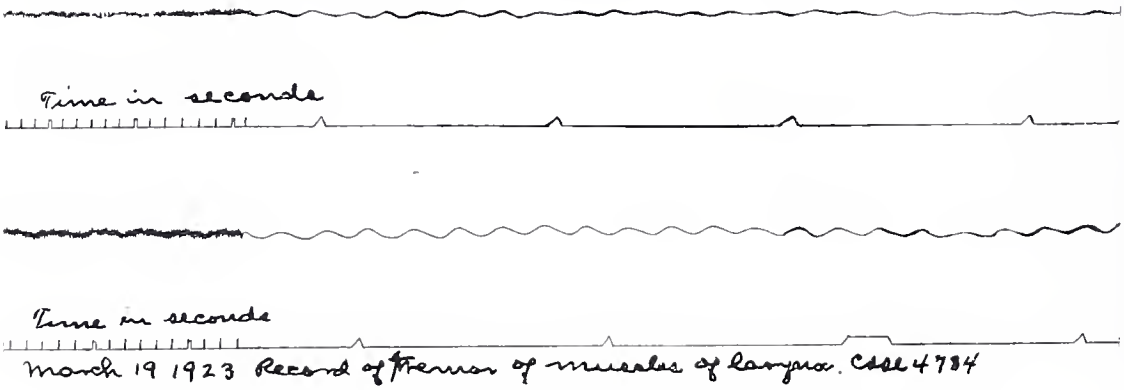

Fig. 5.—Record of tremor of laryngeal muscles (case 5); 6 oscillations each second.

There was a typical parkinsonian syndrome, involving the left side more than the right, and consisting of marked tremor of the tongue, larynx (fig. 5), and lower jaw, rhythmic lateral movements of both vocal cords, synchronous with movements of the lower jaw, eyelids and muscles of the neck, and weakness of the elevators of the soft palate. The spinal fluid Wassermann reaction was negative. Septic tonsils, catarrhal pharyngitis, and two abscessed teeth were found.

Two rabbits injected subdurally with the washings of the nasopharyngeal swab developed marked tremor of the masseters, worse when under tension, sometimes causing the teeth to chatter. One also had rhythmic lateral movements of the head, synchronous with nystagmus in which the left eye rotated upward as the right rotated downward, and vice versa. This animal was found dead the 3rd morning after injection. Subpial hemorrhages were found over the base of the right cerebrum, the pons, and the medulla. Cultures of the blood were sterile; those from the brain yielded a moderate number of the characteristic streptococcus. The tremor of the jaw (fig. 6) persisted for 7 days, when it ceased. The animal was anesthetized on the 11th day after injection. No

gross lesions were found in the brain, and cultures proved sterile. Sections revealed typical lesions in the cortical and subcortical layers of the ganglion cells and in the midbrain beneath the 3rd ventricle.

RESULTS IN THE MYOCLONIC GROUP

Tremors and rhythmic muscular contractions were distressing symptoms, either before or at the time of study, in 17 of the patients. A number had had hiccup. The ages in this group ranged from 2½ to 54 years. The duration of symptoms was 5, 6, and 7 days in one each; 3 weeks in one; 4 weeks in three; 6 weeks in one; 2, 3 and 5 months in one each; and from 1 to 2½ years in six. Eight were females, and 9 were males. A summary of the incidence of the symptoms produced in animals is given in table 1. The incidence of spasms of muscles was higher than in the other types of encephalitis, 35 of 77 animals manifesting this symptom. In many, the spasms were severe

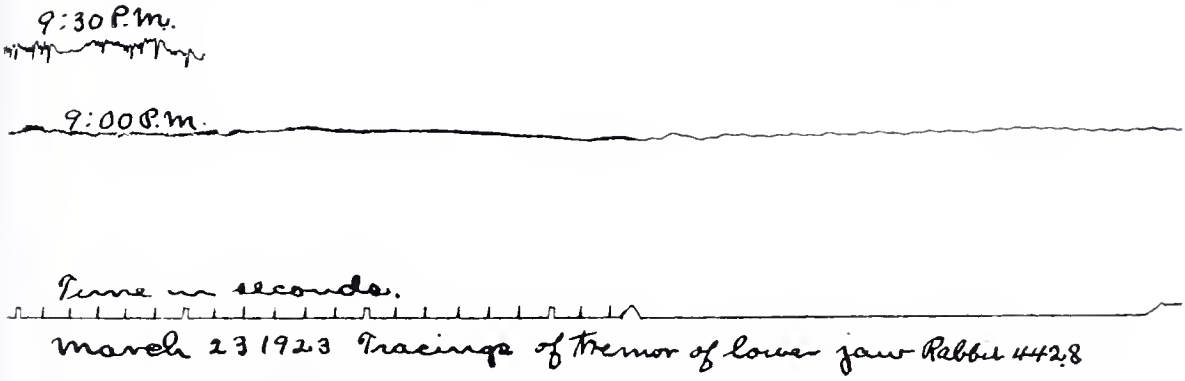

Fig. 6.—Record of tremor of muscles of lower jaw when at rest and when under slight tension, of rabbit 4428, injected with the streptococcus from the patient in case 5; 36 oscillations each second.

and extensive; 6 had spasms of the diaphragm, and 42 had tremor. These findings are in sharp contrast to those in the control series, in which spasms of the muscles developed in only 2, and tremor in 4 of 106 animals injected.

In one case the encephalitis streptococcus which was isolated from the nasopharynx after a superimposed throat infection had subsided produced a myoclonic type of encephalitis in the earlier animal passages, but with increase in virulence from successive rapid animal passage, myoclonus disappeared and meningitis became more marked. Thus, 7 of 13 rabbits injected in the first 4 passages developed tremors or twitchings of muscles, and 4 (30%) developed meningitis, whereas none of the 9 injected in the next 4 passages developed myoclonus, and all had meningitis. The results in this group are illustrated in the following case.

CASE 6.—A man, aged 46, came to the Clinic on March 12, 1923, on account of severe, fairly constant pain in the left lower chest and abdomen, associated with rhythmic contractions of the muscles of the abdomen, which had appeared suddenly 3 months before. At first the attack was extremely severe, and for 5 days was associated with persistent hiccup.

Divergent strabismus, nystagmus, frequently recurring clonic contractions of the muscles of the abdomen, more marked on the left, moderate pyorrhea, 5 teeth with periapical infection, and septic tonsils were found. The spasms of the abdominal muscles disappeared for short intervals when the patient's mind

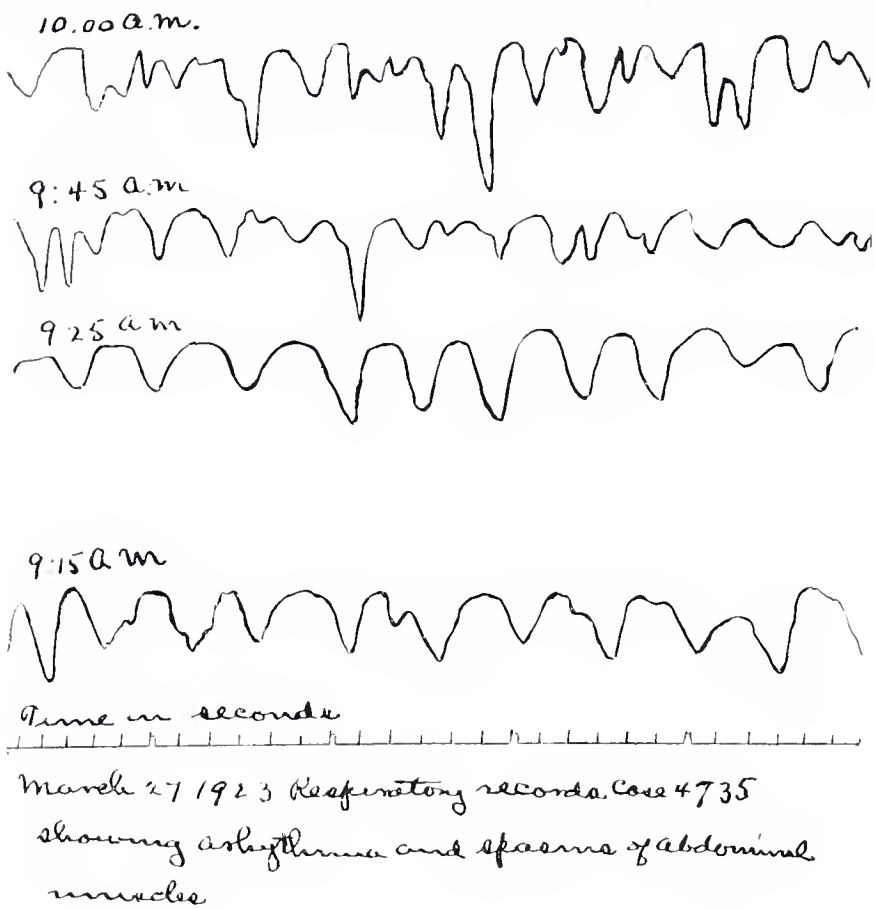

Fig. 7.—Respiratory record (case 6) showing arhythmia, spasms of abdominal muscles, and the effect of diverted attention.

was diverted, as indicated in the tracings (fig. 7). Immediately after placing the tambour around the patient's abdomen (March 27, 9:15 a. m.), and making the necessary adjustments, in which he was much interested, the spasms disappeared. They were absent at 9:25, but at 9:45 they had returned, and by 10 they occurred about as before.

One rabbit was injected intracerebrally with the salt solution suspension of pus expressed from the tonsils. It remained well for 2 days, when it developed hyperpnea, tremor, and twitching of the masseters, and 3 days later,

marked spasms of the abdominal muscles and diaphragm (fig. 8). The spasms of muscles in the rabbit also were susceptible to external influences. When the animal was in a normal position, spasms of the abdominal muscles and diaphragm occurred rhythmically (fig. 8, *1*). When tied to a board with the extremities outstretched, and a solution of procain injected subcutaneously at the ensiform (preparatory to obtaining a tracing of the spasms of the diaphragm), the spasms promptly disappeared (fig. 8, *2*). When the animal was untied and allowed to assume its natural upright posture, the spasms returned (fig. 8, *3*), and again disappeared when tied to the board (fig. 8, *4*). After several repetitions of this

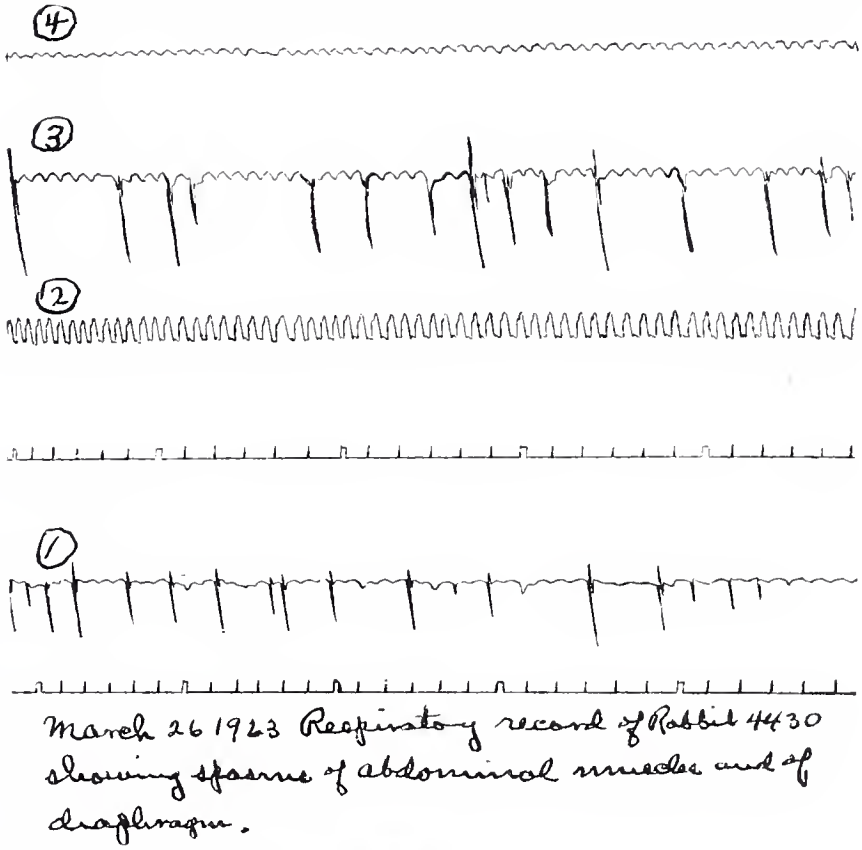

Fig. 8.—Respiratory record of rabbit 4430 injected with the streptococcus from case 6, showing spasms of abdominal muscles and diaphragm, and the effect of change in position and muscular tension.

procedure, the animal was chloroformed. No gross lesions were found; cultures from the brain yielded a large number of the streptococci in pure form; those from the blood were sterile.

RESULTS IN THE MENINGITIC GROUP

Unusual meningeal involvement was evident in 5 patients. All were males, and their ages ranged from 17 to 58 years. The duration of symptoms was 2, 6, 10, and 30 days, and 9 weeks, respectively.

The spinal fluid was turbid in all, and a streptococcus which was agglutinated by my anti-encephalitis and antipoliomyelitis serums was isolated in 3 cases. In a case of meningitis not included in this series, Dr. George H. Wheeler, of the New York Hospital, to whom I am indebted for a subculture of the strain, isolated a pneumococcus which was not agglutinated by the type pneumococcus serums, but was agglutinated specifically by my anti-encephalitis and antipoliomyelitis serums. The incidence of symptoms following the injection of these strains (table 1) was similar to that in the other groups, except that meningitis was more common.

Case 7.—A boy, aged 17, a patient of Dr. Belt of Dodge Center, Minn. About 10 days after an attack of influenza, which was epidemic in his community at the time, the patient developed high fever, pain in the left eye and left side of the head and ear, photophobia, diplopia, and 2 days later extreme restlessness and rigidity of the neck. He was seen in consultation the following day by Dr. Hallenbeck, of the Mayo Clinic, who found weakness of the muscles of the left arm, marked rigidity of the neck, and turbid cerebrospinal fluid under

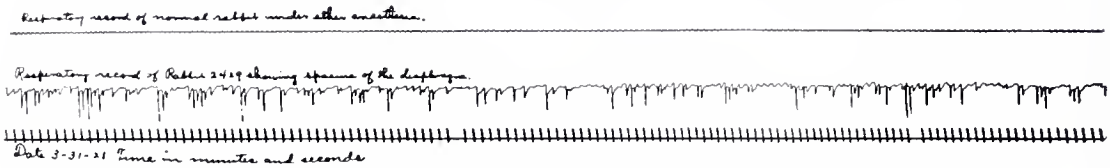

Fig. 9.—Respiratory record of rabbit 2429 injected with strain 4226 in the 24th rapidly made subculture.

increased pressure. There was no tenderness over the mastoids, and the drum membranes were normal.

The spinal fluid contained many extracellular gram-positive diplococci, some in short chains, and many cells, mostly leukocytes. The cultures yielded a pure growth of a streptococcus which resembled that isolated in cases of encephalitis, and it was agglutinated by my anti-encephalitis and antipoliomyelitis serums. I saw the patient the following day, March 18, 1921. He was stuporous, had almost complete paralysis of the left leg, weakness of the right leg, complete loss of reflexes on the left side, and diminished knee jerk and tendo achillis jerk on the right. There were inequality of pupils, external strabismus on the left side, increased respiration, high fever, intermittent hiccup, rapid pulse, and cyanosis. The spinal fluid was still turbid and yielded a pure growth of the streptococcus in large numbers. Twenty c c. were withdrawn, and 20 c c. of the anti-encephalitis serum were given intraspinally and 20 c c. intravenously. Several hours later there was decided improvement, which lasted for 18 hours. The patient grew worse and died the following day. Necropsy was refused.

Experiments.—The effect of the streptococcus from the spinal fluid was tested by injecting 8 rabbits intracerebrally (table 2). Three of these were injected with from 0.1 c c. of a 1 : 100 dilution to 0.2 c c. of the undiluted spinal fluid, two after 2 subcultures, two after 14, and one after 24. Two recovered and 6 died. None had spasms of the diaphragm; 5 had meningitis. Sixteen rabbits were injected with the strain in the 1st passage, after 24 rapidly made

subcultures, together with horse serum. Only one recovered. Eight developed spasms of the diaphragm (fig. 9), 14 spasms of other muscles, and 12 (75%) meningitis. The microscopic lesions found are well illustrated in figures 10, 11, and 12, and the diplococci found in the lesions in figure 13. In the second passage, only 2 rabbits were injected, both with the emulsion of the brain of a rabbit that had spasms of the diaphragm. Both died of meningitis and encephalitis; one had spasms of the diaphragm. Sixteen rabbits received the strain in brain emulsion or in the 45th rapidly made subculture in the 3rd animal passage. Four recovered and 12 died. The incidence of the important symptoms in these 4 groups of animals is summarized in table 2.

The effect of the horse serum known to be toxic was clearly harmful on intracerebral injection. Not only the incidence and range of grave symptoms,

TABLE 2

INCIDENCE ACCORDING TO ANIMAL PASSAGE OF THE MORE IMPORTANT SYMPTOMS IN RABBITS FOLLOWING INTRACEREBRAL INJECTION OF MATERIAL CONTAINING THE STREPTOCOCCUS

Animal Passage	Inoculation	Animals Injected	Mortality, %	Spasms of Muscles			Tremor	Ataxia	Hyperpnea	Paralysis	Lethargy	Meningitis	
				Diaphragm		Other							
				No.	%	No.						No.	%
First	Intracerebral	8	75	0	0	2	6	4	3	5	1	5	62.5
First, with simultaneous injection of horse serum	Intracerebral	16	94	8	50	14	15	6	0	15	2	12	75
Second	Intracerebral	2	100	1	50	1	2	2	1	2	0	2	100
Third	Intracerebral	16	75	2	12.5	8	8	4	1	7	5	9	56
First, second and third	Intravenous, Intraocular, Intranasal	12	67	1	8	4	3	2	1	4	5	4	33

but the mortality rate increased. The strain in the second passage, isolated from the brain of one of the animals receiving normal horse serum besides the 24th subculture, which had succumbed from acute meningo-encephalitis, had increased in virulence, whereas in the 3rd passage the virulence had diminished, the strain having been isolated from the brain of a rabbit, 5 days after injection in the anterior chamber of the eye, and from the brain of a monkey 5 days after the nose had been packed with gauze soaked in the culture. The power to produce spasms of muscles, tremors, and paralysis had greatly diminished, the incidence dropping from 67 to 31%, 87 to 50%, and 83 to 44%, respectively, whereas the incidence of lethargy increased from 12.5 to 31%.

The localizing power of the strain in the 2d passage was further tested by injecting the filtrate of the emulsion of the brain of positive rabbits intracerebrally into 2 rabbits, and into the anterior chamber of the eyes of 2 rabbits,

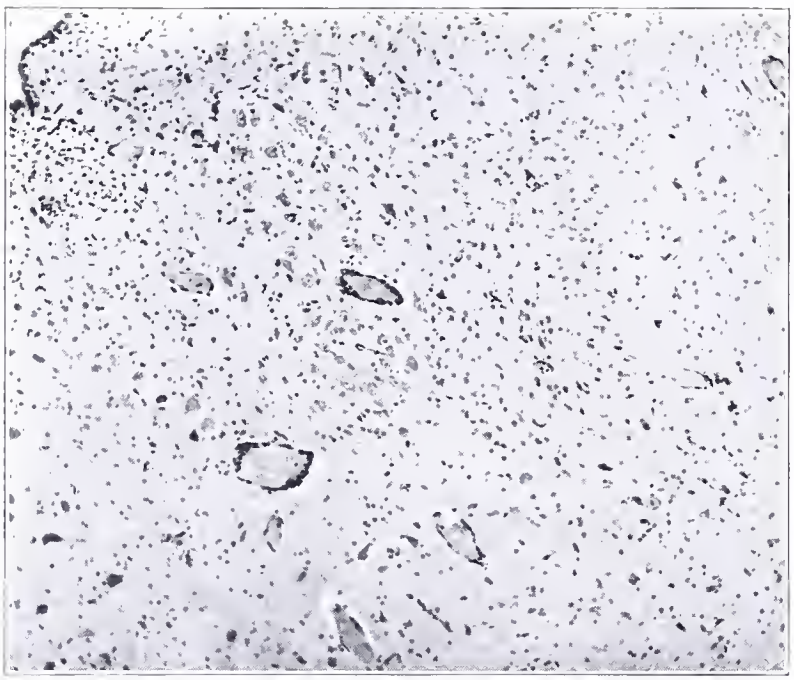

Fig. 10.—Localized and perivascular infiltration in the medulla of rabbit 2429. Hematoxylin and eosin (× 60).

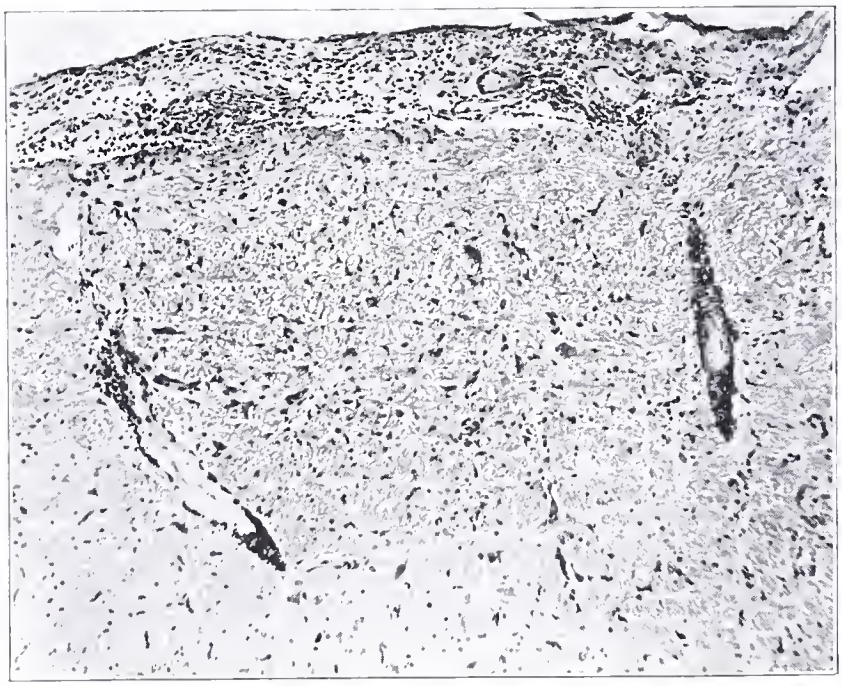

Fig. 11.—Infiltration of the pia over the anterior aspect of the medulla, and perivascular infiltration in rabbit 2386 (case 7, strain 4226). Hematoxylin and eosin (× 60).

and by packing the nose of a monkey with gauze soaked in the culture. The 5 animals developed symptoms of meningo-encephalitis. The second culture from a filtrate of the same brain emulsion was injected intravenously into 4 rabbits and intraperitoneally into 1, and into 2 rabbits by packing the nose with gauze soaked in the culture. All but 2 of these rabbits developed symptoms. One of the rabbits injected intravenously and one whose nose was packed remained well.

Monkey 303 was injected intracerebrally, on April 5, 1921, with 0.5 c c. of sterile glucose-brain broth, and the nose was packed with gauze soaked in a dense suspension of the streptococcus from the spinal fluid after 24 rapidly made subcultures and 1 animal passage. The following day the animal appeared well, and the gauze was removed from the nose. On April 7, it was hyperirritable and had developed slight weakness of the extremities. On April 8, there were rapid vertical nystagmus, internal strabismus of the left eye, high fever, tremor of the head, and marked weakness of the muscles of the extremities, especially of the extensors. On April 9, the animal was more quiet. The hind extremities were completely paralyzed, and the fore extremities were extremely weak; the nystagmus was less marked. On April 10, the animal was found dead.

Necropsy revealed marked congestion of the vessels of the meninges, clouding and infiltration of the pia over the pons, medulla, and upper cervical cord, and a moderate number of small hemorrhages in the medulla. Cultures from the ventricular fluid yielded innumerable colonies of the characteristic streptococcus.

Filtrates of the emulsions of the brain of 2 rabbits injected with the strain in the second passage were injected into 7 rabbits. Three developed symptoms, and from the brain of each the characteristic streptococcus was isolated.

Three rabbits were injected intravenously with the strain in the first animal passage, after facilitating localization by injecting 0.5 c c. of sterile broth intracerebrally. One remained well, the other 2 developed characteristic symptoms of encephalitis, illustrated in the following protocol.

Rabbit 2386 was injected intracerebrally on March 18, 1921, with 0.5 c c. of sterile glucose-brain broth and intravenously with 2.5 c c. of the spinal fluid containing large numbers of viable streptococci. It remained well until the 2d day, when tremor of the muscles of the fore extremities and reluctance to move were noted. On March 21, it sat quietly, with fine tremor of the ears, marked tremor of the entire body, which became worse on exertion, decided weakness of the hind extremities, and increased respirations. On March 22, twitching and tremor of all muscles were severe, and when the animal attempted to hop, marked clonic spasms of the muscles of the extremities developed. On March 23, at 6:45 a. m., it was found lying on its side with almost continual tremor, and clonic twitching of the muscles of the extremities and of the thorax. At 9 a. m., marked twitchings of the muscles of the abdominal wall, and extreme hyperesthesia had developed. At 4 p. m., the condition was worse, and while under observation the animal died in a series of convulsive spasms. Irritability of most of the muscles disappeared promptly after death, but that of the abdominal muscles persisted for 5 minutes. Pricking or slight pressure provoked sharp clonic spasms for a time, but they gradually grew weaker, even though the stimulus was increased in severity.

Necropsy disclosed distinctly turbid cerebrospinal fluid, marked edema and infiltration of the pia on the anterior aspect of the medulla and upper 2 cm. of the cervical cord, several large hemorrhagic areas in the upper cervical cord, and hemorrhagic edema surrounding the nerve trunk of the inner aspect of the foreleg. Cultures from the brain and spinal fluid revealed a pure growth

of the streptococcus; those from the blood, liver, kidney, and urine remained sterile. Sections of the brain revealed localized and perivascular round-cell infiltration, most marked in the pia over the medulla and in the medulla (figs. 11 and 12).

Five of 8 guinea-pigs injected intraperitoneally with the strain in the 24th rapidly made subculture died. None of these showed definite peritonitis, and the cause of death was not apparent at necropsy. Microscopic examination of 2 that died 11 and 4 days after injection revealed localized perivascular infiltration in the region of the midbrain.

RESULTS OF INTRAPERITONEAL INJECTION INTO MICE

The results following intraperitoneal injection into mice are best considered in connection with the protection experiments, summarized in table 3.

TABLE 3

RESULTS OF PROTECTION AND IMMUNIZATION EXPERIMENTS IN MICE FOLLOWING INTRAPERITONEAL INJECTION OF THE STREPTOCOCCUS

Animal Passage		Treatment	Injected	Died	Mortality, %
First		None	15	8	53
Second	Primary culture	None	9	9	100
		Normal horse serum	9	8	89
		Immune horse serum	9	6	67
	Twenty-fourth subculture	None	4	4	100
		Normal horse serum	4	3	75
		Immune horse serum	4	1	25
Third, second subculture		None	6	4	67
		Previous intraperitoneal immunization with the heat-killed organisms	6	0	0
		Previous subcutaneous immunization with the heat-killed organisms	6	1	17

The dose varied from 0.5 to 1.5 c c. of the glucose-brain-broth culture. The virulence of this meningitis strain was higher than that of the strains from chronic cases of encephalitis. Eight of 15 mice injected with the strain as isolated died from peritonitis.

The first group of 27 mice injected with the strain in the 2d animal passage (table 3) received the primary or 24th rapidly made subculture in glucose-brain broth. The dose of the culture was comparable for the controls and for the mice treated with serum. In this series, 0.5 c c. of the culture was incubated with an equal amount of the serum for 2½ hours before injection. It will be noted that the normal horse serum had a slight, and the immune horse serum a more marked protective effect, the mortality for the untreated mice being 100%, as compared with 89% for those treated with normal horse serum and 67% for those treated with the encephalitis serum.

In the second series of protection experiments, the culture was injected intraperitoneally, and the serum (0.2 c c.) subcutaneously immediately afterward. Again the normal horse serum seemed to have a slight, but the immune horse serum a greater protective power. The mortality was 100% for the controls, and 75 and 25%, respectively, for the animals treated with serum.

The streptococcus, after one animal passage, was grown in glucose-broth bottles and a vaccine prepared by heating the dense sodium chloride solution suspension to 60 C. for 45 minutes. Mice were immunized by injecting intraperitoneally and subcutaneously increasing doses of the heated organisms on 3 successive days each week for 4 weeks. The dose ranged from 90,000 to 1,350,000 of the dead bacteria. The test injection, made 4 days after the last vaccination, consisted of 1 and 1.5 c c. of the glucose-brain broth culture after 3 animal

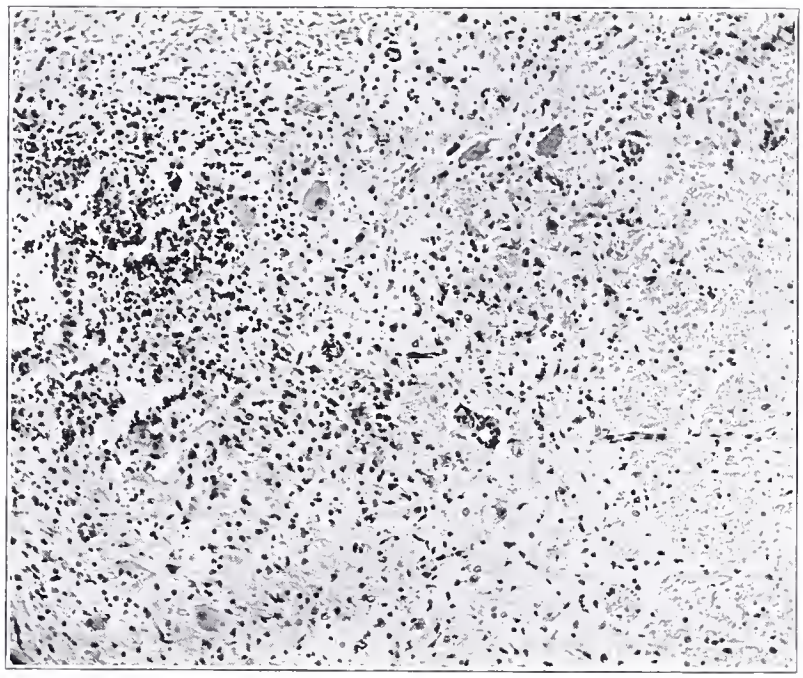

Fig. 12.—Localized and perivascular infiltration in the medulla of rabbit 2386. Hematoxylin and eosin (× 75).

passages. Four of the 6 controls died, none of the 6 immunized by intraperitoneal injection died, and only 1 of the 6 immunized by subcutaneous injection, a mortality of 67 and 17%, respectively.

Of 22 untreated mice that died following injection of this strain in the 1st, 2d, and 3rd animal passages, only 13 showed gross evidence of peritonitis. The streptococcus was isolated from the peritoneal fluid in 15, from the blood in 16 and from the brain in 14. In 5 of the 7 animals in which the peritoneal fluid was sterile, cultures from the brain yielded the characteristic streptococcus. Because of these findings, the brains of some of the mice were placed in formalin for microscopic study. All of 10 injected with the strain in the second animal passage, either in the primary culture or the 24th subculture, died, one 24 hours after injection, three in 2 days, one each in 4, 5, 6, 7, 8, and 10 days,

after injection. Cultures from the peritoneum were sterile in 5; cultures from the brain were positive in all, and from the blood in all but 1, which died 10 days after injection. Marked leukocytic infiltration of the meninges in localized areas and leukocytic and round-cell infiltration around vessels in the subcortical and midbrain region were found in 4, and slight changes in 5, whereas in 2, no changes were found.

Comment.—Characteristic localization followed intracerebral, intravenous, and intraperitoneal injections, inoculation of the anterior chamber of the eye, and packing of the nose with gauze soaked in the culture. It occurred after as many as 69 rapidly made subcultures, and through 3 animal passages. Intracerebral inoculation of filtrates of brain emul-

Fig. 13.—Diplococci in the lesions of rabbits 2429 and 2386; shown in figs. 10, 11, and 12. Gram (× 1000).

sions and cultures of the streptococcus from a filtrate also gave positive results.

In the first two animal passages the reaction incited in the brain and meninges was almost wholly polymorphonuclear, even in animals that died some time after inoculation. In the 3rd passage, after the strain had been in the brain 5 days after inoculating the anterior chamber of the eye of a rabbit and packing the nose of a monkey, and had lost in virulence, leukocytic reaction and meningitis were less pronounced, and perivascular and parenchymatous infiltration, with a preponderance of round cells, were the dominating picture.

If these studies had not been made, this case would have been reported as streptococcic meningitis. The localizing power of the streptococcus isolated, its specific agglutination by the serum prepared with a similar streptococcus, but from a case of lethargic encephalitis, and the fact that this serum afforded protection in mice, indicate that the case was definitely related to epidemic encephalitis, but because of the unusually high virulence of the organism, meningitis dominated the picture.

RESULTS IN THE MIXED OR UNCLASSIFIED GROUP

The symptoms of 31 patients were so varied that it was difficult to determine the most prominent; therefore they were placed in the mixed or unclassified group. The age of the patients ranged from 2 to 64 years. The duration of symptoms was two weeks or less in 12; from one to six months in 10; and from six months to two years in 9.

Spasms and tremor of muscles, paralysis of groups of muscles, dizziness, ataxia, and increased respiration were common symptoms. A history of previous influenzal or other infection was given in 15 cases; in the others, the attack began from no apparent cause. Focal infections in teeth, tonsils, or sinuses were pronounced in most instances, and catarrhal pharyngitis was present in all. A history of previous illnesses, probably focal in origin, was unusually common. The incidence of symptoms in animals (table 1) roughly paralleled those in the patients. In a number the clinical picture resembled that of poliomyelitis (cases 8 and 9).

CASE 8.—A man, aged 23, came to the Clinic on Feb. 23, 1923. The night before, while reading, he had developed headache, dizziness, and diplopia. The day of admission he also had ptosis of the right upper eyelid, with fine twitchings, a slight chill, severe headache over the right frontal region, slight staggering, and fever. The patient was restless; the pupils were unequal, the left being larger; the knee jerks were exaggerated; the leukocytes numbered 13,600; the Kolmer reaction was negative; the temperature was 100; and the spinal fluid contained 1 lymphocyte for each cubic millimeter.

The patient was sent to the hospital, where he grew rapidly worse. Respirations became rapid (from 50 to 60 a minute) and extremely irregular in rate and amplitude. The pupils became fixed, and nystagmoid jerks of the eyeballs, and edema of the eyelids and lips developed. The temperature remained at about 100 F. for 3 days, then rose to 103 F., as respiration became extremely rapid, irregular and labored, and death followed from respiratory failure.

Necropsy revealed patchy areas of hemorrhagic bronchopneumonia, gelatinous mucopurulent material in the sphenoid and ethmoid sinuses, and extreme congestion of the vessels of the meninges, brain, and other viscera. Sections of the medulla revealed typical lesions (fig. 14) containing the streptococcus (fig. 15); and no bacteria could be found in the parts of the brain and medulla which were free from lesions.

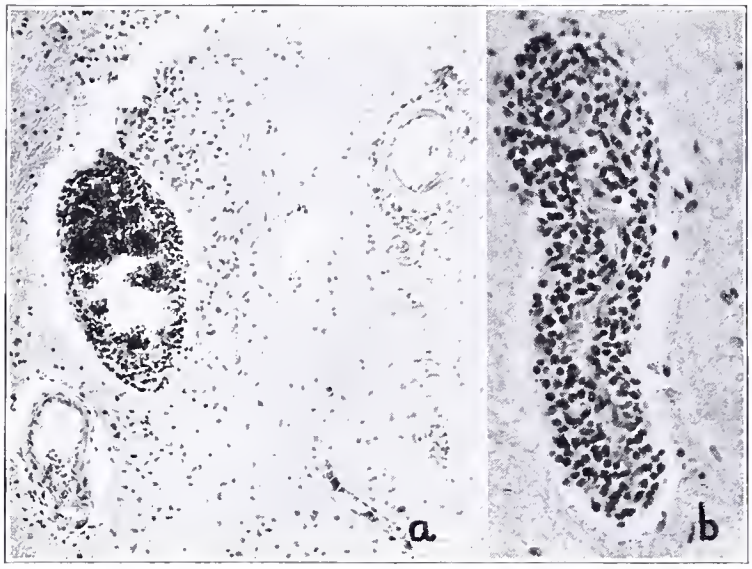

Fig. 14.—Localized and perivascular round-cell infiltration in medulla (*A*) and pons (*B*) of case 8. Hematoxylin and eosin (× 80 and × 120).

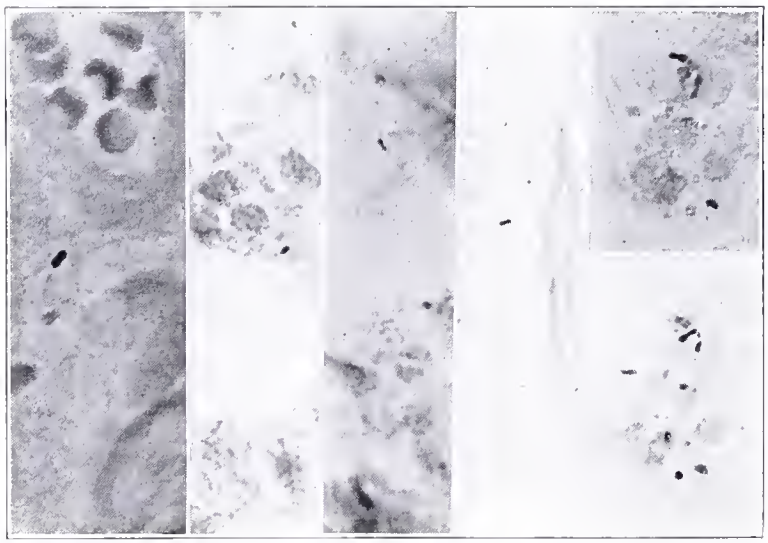

Fig. 15.—Diplococci of varying size adjacent to dilated capillaries and in lesions in the medulla of case 8. Note wide variation in size and the extremely small chains in the figure below and to the right. Gram (× 1000).

The sodium chloride suspension of nasopharyngeal washings obtained on Feb. 24 was without effect on 2 rabbits injected; that obtained 2 days later produced a bulbar type of encephalitis in one of two rabbits injected; the other remained well. The blood-agar plate made directly from the nasopharyngeal suspension revealed numerous colonies resembling the encephalitis streptococcus. Transfer to glucose-brain broth from a single colony was made, and 2 rabbits were injected intracerebrally. Both developed encephalitis, with irregular and increased respiration as a prominent symptom.

A streptococcus, apparently identical with that isolated from the throat directly and from the brains of the animals that developed symptoms, was

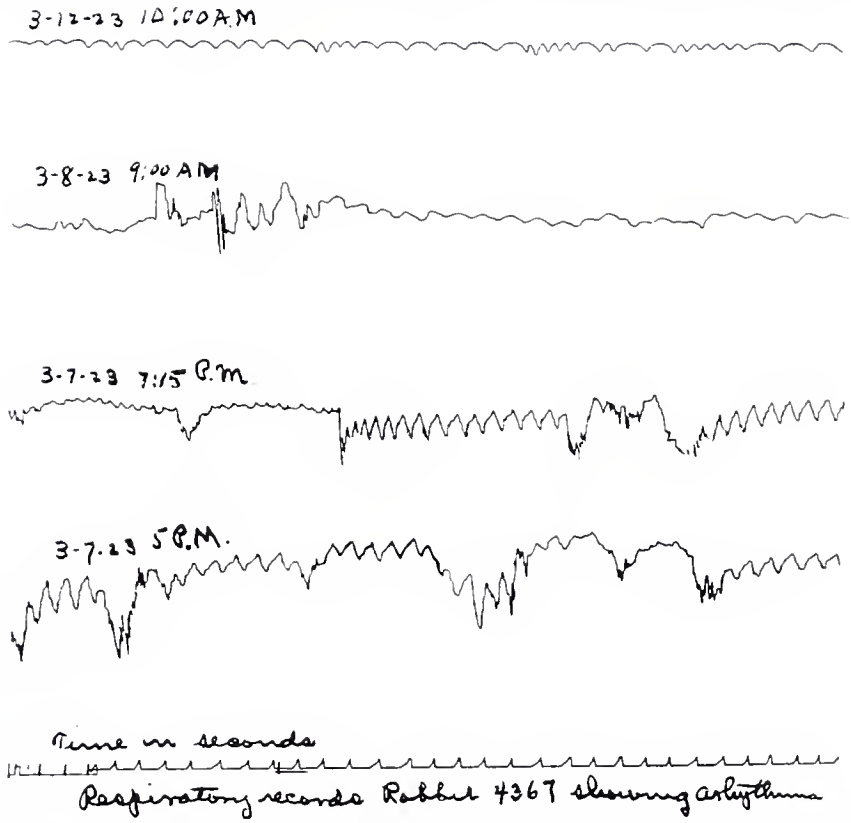

Fig. 16.—Respiratory record of rabbit 4367 (case 8) showing marked arhythmia, 1, 2, and 6 days after subdural injection of the streptococcus isolated from the medulla after 24 rapidly made subcultures.

isolated, after death, from the medulla and from the areas of hemorrhagic edema in the lung. The effect of the strain from the medulla and the lung was tested under identical conditions in rabbits, guinea pigs, and mice. Both strains were rapidly subcultivated through 24 culture generations, and 1 rabbit was injected with 0.2 c c. of the undiluted, and one with 0.2 c c. of a 1 : 100 dilution of a glucose-brain broth culture of each strain. All developed symptoms of encephalitis, with marked involvement of respiration. The degree of disturbance in respiration is illustrated in fig. 16, and peculiar movements of the head, which developed as a late manifestation, in fig. 17. The one receiving the large dose

Fig. 17.—Peculiar tilting of the head in rabbit 4367 ten days after acute symptoms had subsided.

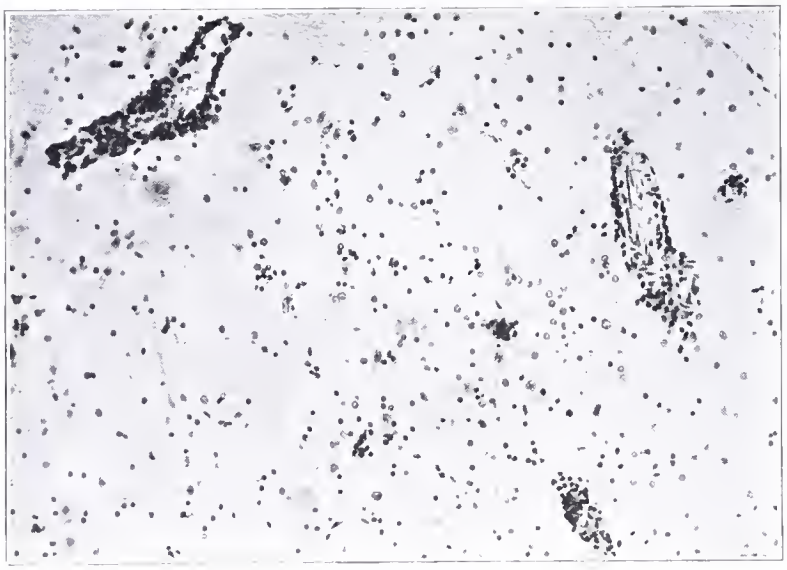

Fig. 18.—Perivascular round-cell infiltration in the midbrain of rabbit 4367.

of each strain developed spasms of muscles, and all revealed marked microscopic lesions in the medulla or midbrain (fig. 18), and diplococci in the lesions (fig. 19).

Of 8 rabbits injected with material from the throat, hyperpnea or arhythmia developed in 6, tremor in 6, restlessness in 5, ataxia in 4, spasms of muscles in 3, spasms of the diaphragm in 1, rhythmic movements in 2, rotary spasm of the muscles of the head in 2, and nystagmus and paralysis in 1 each. Two rabbits were injected with the emulsion of the brain; both remained well. Two of 3 injected with the emulsion of the pons and medulla remained well, whereas 1 developed peculiar irregular respirations 2 days after injection (fig. 20) and slight turning of the head in 9 days, but no other symptoms. All were chloroformed 16 days after injection, and cultures proved sterile in all. Emulsions of the brain of the rabbit which developed arhythmia were injected into 2 rabbits; both remained free from symptoms. The spinal fluid, the filtrates of

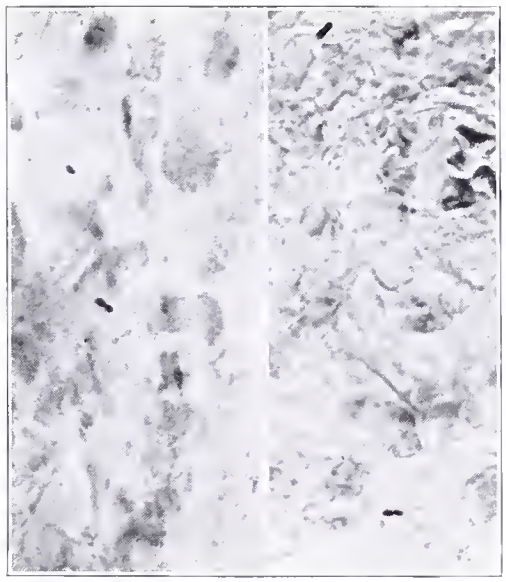

Fig. 19.—Diplococci in the lesions of the medulla of rabbit 4369, injected 4 days previously with the streptococcus from the medulla of the patient in case 8.

nasopharyngeal washings, and emulsions of the pons and of the medulla were without effect in 11 rabbits injected.

Four guinea-pigs were injected intratracheally with the strains from the medulla and the lung, 2 in the 24th rapidly made subculture before, and 2 after 1 mouse passage. Only slight symptoms developed; 2 of the animals injected with the strain from the medulla and 2 with that from the lung developed noteworthy respiratory symptoms; none died. Reduction in leukocytes was slight. One animal in which respiratory symptoms persisted was chloroformed 23 days later, and serofibrinous and adhesive pleuritis and bronchopneumonia of the right lung were found. A pure culture of the streptococcus was isolated from the pleural fluid; the blood proved sterile.

Four mice were injected intraperitoneally with 1.5 c c. of glucose-blood-broth culture in the 24th rapidly made subculture of the strains, respectively, from the medulla and the lung. One injected with the former, and 2 with the latter

died in 48 hours, not from peritonitis but from pleuritis, hemorrhagic edema of the lung, and marked degeneration of the liver. The streptococcus was found in the blood of each in moderate number, in the pipetted material from the brain in large number, and in the turbid pleural fluid in infinite number. The strain from the mouse injected with the streptococcus from the medulla was then passed successively through mice in series of 2 or 4, for 8 passages. The mortality increased somewhat, but the cultural characteristics and the morphology of the strain, and the findings in the mice throughout the series, were almost identical with those noted after the 1st animal passage. In every instance, mice that died early showed pleuritis, hemorrhagic edema, or bronchopneumonia, always with slight or no evidence of peritonitis, and often with localization in the brain.

The effect of the strain on intratracheal injection in guinea-pigs after 9 successive passages in mice was practically identical with that before and after

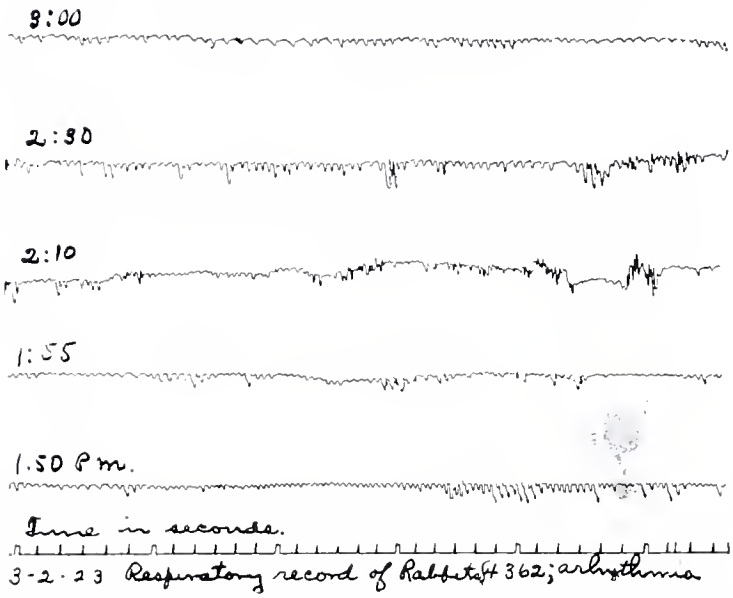

Fig. 20.—Respiratory record of rabbit 4362 showing arhythmia 2 days after injection of the emulsion of the medulla and brain of the patient in case 8.

1 mouse passage, and, what is more significant, its agglutinating property remained unchanged throughout. The patient's own serum agglutinated specifically the streptococcus isolated from the nasopharynx and medulla and the streptococcus from other cases of encephalitis.

Comment.—The points deserving emphasis in this case are the pronounced evidence of involvement of the respiratory center; the isolation of the streptococcus from the medulla; the marked lesions in the medulla, in which the streptococcus was demonstrated; the striking occurrence in animals of respiratory arhythmia following injection of the emulsion of the medulla (fig. 20) and the cultures of the streptococcus, even after 24 rapidly made subcultures (fig. 16); the high inci-

dence of movements of the head; the complete absence of symptoms following injection of the spinal fluid and filtrates of the nasopharyngeal washings and emulsion of the medulla; the tendency of the strain to produce pleuritis, edema of the lungs, and lesions of the brain when injected intraperitoneally into mice, and mild lesions of the lung when injected intratracheally into guinea-pigs; the unchanged virulence and agglutinating properties following successive animal passages, and the agglutinating power of the serum of the patient over this and other encephalitis strains.

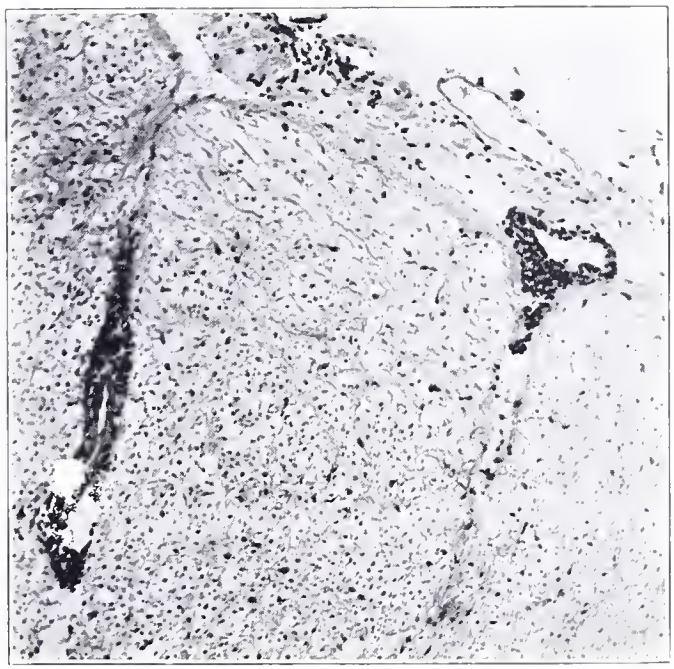

Fig. 21.—Localized and perivascular infiltration in the pia and pons (case 9). Note the similarity of the lesions to those in the medulla of rabbit 2386 shown in fig. 11. Hematoxylin and eosin (× 100).

CASE 9.—A girl, aged 17, was brought to the hospital on March 30, 1923, on account of difficulty in breathing, which developed that day and from which she died 10 hours after admission. She had been well until 10 days before, when she had to leave school on account of pain in the left eye, the left side of the face, and the left ear. The pain had continued with little interruption for 6 days, when it grew worse, and the lower part of the face began to twitch; 2 days later paralysis of the muscles of the face, and diplopia developed.

A masklike expression, drooping eyelids, twitchings of the muscles of the chin, transitory hiccup, sluggish reflexes of the anus and legs, and nystagmus were noted. The pulse was 154 and respirations 60. The patient became comatose soon after admission, the temperature rose to 105 F., and she died, apparently from failure of the respiratory center.

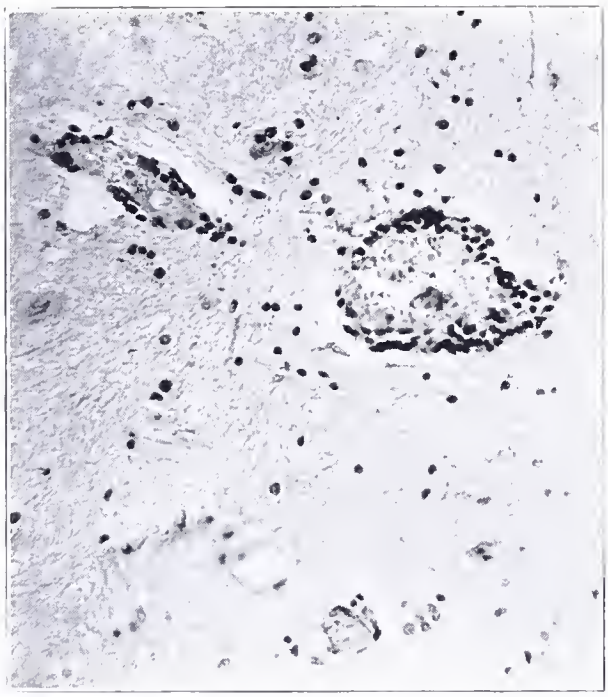

Fig. 22.—Perivascular infiltration in the medulla (case 9). Hematoxylin and eosin (× 150).

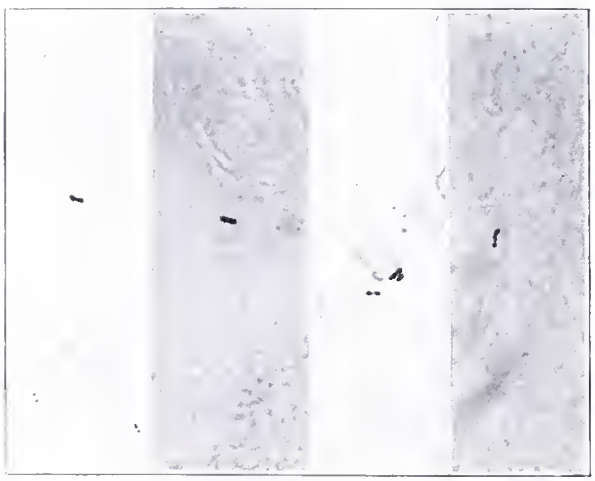

Fig. 23.—Diplococci of varying size in the lesions shown in figs. 21 and 22. Gram (× 1000).

Necropsy revealed congestion of the vessels of the meninges and brain. The lungs were normal. Cultures from the nasopharyngeal washings yielded a large number of green-producing streptococci in almost pure form. Those from the medulla, and from the filtrate of an emulsion of the medulla and brain yielded a pure culture of a similar streptococcus. Sections revealed typical lesions in the medulla and pons (figs. 21 and 22), in which the streptococcus was demonstrated (fig. 23).

Four rabbits were injected subdurally with the washings from the nasopharyngeal swab. Two died during the night, and no symptoms were noted; the other 2 developed markedly increased and irregular respiration, nystagmus, tremor and twitching of the muscles, and weakness, beginning in the fore extremities; one died in 2, the other in 4 days. Cultures from the blood were sterile; those from the brain yielded a streptococcus identical with that isolated from the medulla and throat of the patient.

The primary culture from the filtrate of the emulsion of the medulla and brain was injected subdurally into 2 rabbits. Both died of rapidly fatal bulbar encephalitis. The respiration became rapid and irregular, and marked tremor, twitching, and weakness of the masseters and muscles of the neck and fore extremities developed in both. Cultures from the blood yielded a few, and those from the brain many, colonies of the streptococcus.

The emulsion of the medulla and the brain was without effect in 4 rabbits. One died in 10 days, and cultures from the brain yielded the streptococcus. A filtrate of the emulsion of the medulla and brain was injected into 4 rabbits; all remained well. The 7 were anesthetized one month later. Cultures from the brain were sterile in all, and no lesions were found in sections.

The streptococcus from the filtrate of the emulsion of the medulla and brain was injected intraperitoneally into 2 mice. Both died within 48 hours without peritonitis or pleuritis, but with invasion of the blood and brain. The streptococcus from the brain of one of these was injected into 4 mice. Two died without peritonitis or pleuritis, and 2 remained well.

Comment.—The absence of previous influenzal or other infection, the rapidly fatal course of the disease in the patient, without lesions in the lung, the presence of typical lesions and of the streptococcus in the pons and medulla, the isolation of the streptococcus from the throat and from the medulla, with which similar symptoms were produced in rabbits, and the absence of pleuritis or edema of the lung following intraperitoneal injection in mice (in sharp contrast to strain 4719), are the points of especial interest in this case. The attack occurred at a time when influenza was prevalent in the school which the patient attended, and when several cases of encephalitis had developed in the community in which she lived.

SUMMARY OF RESULTS WITH REGARD TO SYMPTOMS, MORTALITY, AND CULTURES

The relative incidence of the characteristic symptoms in the various forms of encephalitis was pointed out in the respective groups. The total incidence of the different symptoms in the encephalitis experi-

ments (table 1) corresponds, in general, to that observed clinically. Thus, of 519 rabbits injected, 115 developed spasms of muscles, 158 ataxia, 220 tremor, 125 hyperpnea, 169 paralysis, and 67 lethargy, approximately the same proportion noted in man in epidemics of this disease. Moreover, the incidence of the more rare symptoms of encephalitis as it occurs in man is paralleled roughly by the incidence in the animals. Thus, spasms of the diaphragm occurred in 25, spasms of the abdominal muscles in 40, nystagmus in 40, rhythmic and tic-like movements in 11 and 5 instances, respectively, turning of the head in 39, retraction of the head in 42, undue restlessness in 57, and convulsions in 7. The incidence in the encephalitis contact experiments, while less marked, was similar. These findings are in sharp contrast to those following injection of 106 animals with material from the nasopharynx of 77 normal controls, when spasms of the diaphragm or abdominal muscles, and lethargy did not develop in a single instance, spasms of muscles occurred in only 2, tremor in only 4, paralysis in 8, ataxia in 17, nystagmus in 3, and retraction of the head in 2.

The data in the table do not adequately express the extremely specific effects of some of the strains. The symptoms were usually more marked and more numerous in the encephalitis than in the normal control series. They often resembled closely in distribution and type those noted in the patient from whom the particular strain was isolated (figs. 2*a* and *b*, 5 and 6, 7 and 8, 9, 16, and 20). In some instances, especially following injection of strains from wildly delirious or maniacal patients and, in rare instances, of other strains after a series of animal passages and many rapidly made subcultures, the symptoms were extremely stormy, and occasionally definitely maniacal. This occurred after intracerebral and other methods of injection of living organisms, and, more rarely, after injection of toxic filtrates and the corresponding dead bacteria.

Illustrative Protocols

Guinea-pig 1324 was injected intratracheally on April 7, 1919, with 1.5 cc. of a glucose-brain-broth culture of the pus expressed from the tonsils of a patient with acute encephalitis, delirium, occupational delusions, and mental confusion. The respirations were moderately increased the day after injection. On April 9, the respirations were less marked, but the animal was slightly ataxic. On April 10, the respirations were slow, the extremities were cold, and the animal was restless, moved constantly, buried its head in the straw on the floor of the cage, and pushed with its nose against the wall of the cage or against its mates. Ataxia, weakness of the left hind leg, and clonic spasms and twitchings of the muscles had developed. It died that night. Necropsy revealed healing bronchopneumonia, and marked congestion of the vessels of the

meninges. Cultures from the brain yielded the streptococcus in pure form; those from the area of consolidation yielded the same organism and staphylococci; the blood was sterile. Two rabbits injected intracerebrally with the streptococcus from the brain developed marked restlessness, tremor, ataxia and weakness of the fore extremities, but recovered. Two guinea-pigs injected intratracheally remained well.

Rabbit 3647 was injected intracerebrally on April 13, 1922, with 1.4 c c. of the filtrate of an actively growing glucose-brain-broth culture of a streptococcus from epidemic hiccup after 2 animal passages and 36 rapidly made subcultures. It remained well until April 19, when it became extremely excitable. It jumped about at irregular intervals, dashing over its mates and bumping its head against the side of the cage, then stood quiet but tense for a short time, breathing rapidly, and looking about as if frightened, with eyes wide open, head high, and ears erect, only again to jump violently at the slightest noise or movement. Impairment of sight was evident, for the animal fell from the open cage, and bumped its nose against its mates or the wall of the cage repeatedly. When grasped gently over the back it cried out, apparently not from pain but from fright. After several days, these symptoms gradually subsided, and the animal appeared well for 5 weeks, when it was found dead. No gross nor microscopic lesions were found, and cultures were negative.

In certain strains, little change in localizing power occurred following animal passage (cases 1, 8, and 9). Certain symptoms developed not only in a particular group of animals, but in series through one or more animal passages. The specific localizing power of other strains was not so stable, and marked changes in symptoms occurred when the strain was passed rapidly through animals (case 7).

Certain symptoms induced experimentally varied greatly. Thus, lethargy appeared in varying degrees of intensity and was readily differentiated from comatose symptoms. In the lethargic condition the animal appeared well except for the abnormal sleepiness, and assumed an almost constant characteristic attitude, with ears erect, head forward, and eyes half closed (fig. 2). In the comatose state, the ears and extremities were relaxed, the animal lay on its side and appeared very sick. Paralysis varied greatly in extent and nature, but in most instances it was flaccid, and usually began in the fore extremities and the muscles of the neck.

Variations in the nystagmus were noted in the animals as in the patients. It was horizontal or vertical, continuous or intermittent, rapid or slow. When slow, synchronous movements of the head or muscular spasms occurred.

Myoclonic twitchings and spasms in the animals resembled those in patients in distribution, severity, and time of occurrence. In some they were brief and rapid, occurring in explosive waves at irregular intervals (fig. 8); in others, rhythmic, often synchronous with respira-

tion, and affecting the musculature of the extremities, face, trunk, and diaphragm. Opisthotonic convulsions and choreiform twitchings also occurred. Muscular rigidity (fig. 2 *b*), especially of the masseters, ocular ptosis, lacrimation, disturbances of vision, urinary retention, profuse salivation, difficulty in swallowing, and stretching, yawning, and sneezing attacks were all noted in certain instances.

The time of development and the duration of symptoms following injection varied greatly, depending on the size of the dose and the virulence of the strain. Symptoms usually developed within a week or 10 days or not at all. Those that survived the acute attack often developed such symptoms as peculiar movements of the head (fig. 17) or abnormal posture, generally regarded as sequelae, and often associated with marked changes in behavior. In some animals, particularly in those injected with strains from acute bulbar types of the disease, rapidly fatal symptoms appeared soon after injection, of which difficult breathing, hyperpnea, and a rapidly progressing weakness were the most striking.

The general neurotropic properties of the encephalitis strains compared with similar streptococci from the throat of normal controls are also shown by the mortality rate (table 1), which in the encephalitis experiments was 74%, in the encephalitis contacts 40%, and in normal controls 23%. Moreover, the cultures showed a higher incidence of green-producing streptococci in the brain and blood of animals injected with encephalitis streptococci. Thus, it was 260 and 89, respectively, of 376 animals cultured in the encephalitis series, 8 and 2 or 29 in the encephalitis contact series, and 24 and 7, respectively, of 97 in the normal control series.

Cultures were made in 514 animals injected intracerebrally or intravenously with material from portals of entry, or with the streptococcus from these, and from the brain and cord in encephalitis. The green-producing streptococcus was isolated from the brain in 281, staphylococcus in 48, hemolytic streptococcus in 18, and gram-negative bacilli in 65. Cultures of the blood yielded the green-producing streptococcus in 94, the staphylococcus in 23, the hemolytic streptococcus in 11, and gram-negative bacilli in 53.

The incidence of the green-producing streptococcus in brain and blood was highest in experiments of short duration and became progressively less as the duration of the experiment became longer. Of 190 cultured within 48 hours after injection, 153 (80%) yielded the streptococcus in the brain, and 55 in the blood; of 123 cultured

from 3 to 5 days after injection, 81 (66%) yielded the streptococcus in the brain, and 34 in the blood; of 59 cultured from 6 to 10 days after injection, 18 (30%) yielded the streptococcus in the brain, and only 2 in the blood, and of 142 cultured 11 days or longer after injection, only 29 (20%) yielded the streptococcus in the brain and 3 in the blood. Cultures of the brain and blood of 113 animals that remained free from symptoms after injection with material from the throat yielded the green-producing streptococcus in only 14 and 5, the staphylococcus in 5 and 2, the hemolytic streptococcus in 5 and 1, and other bacteria in 14 and 10 instances, respectively.

The significance of the isolation of the streptococcus is emphasized by the fact that streptococci were isolated in only 24 of 150 negative animals injected with inert filtrates, emulsions of brain, sodium chloride solutions, broth, highly diluted cultures, and cultures that had lost their virulence. In each of the 24 from which it was obtained, material known to contain the streptococcus had been injected; moreover, streptococci were isolated from the brain of only 12 of 105 rabbits, anesthetized in from 2 to 4 days after intravenous injection of streptococci from sources other than diseases of the nervous system, and finally the streptococcus was not isolated from the brain in a single instance in 60 uninoculated rabbits anesthetized as controls.

SUMMARY OF GROSS AND MICROSCOPIC CHANGES

The findings at necropsy in the 5 patients who died were largely negative. Slight clouding of the pia over the medulla was found in 2 patients with bulbar symptoms. Microscopic changes, while varying in extent and degree, were typical in each instance. They were chiefly perivascular and round-cell infiltration (figs. 14, 21, and 22), most marked in the basal nuclei in the midbrain region, and in the pia over the anterior aspect of the medulla and pons (fig. 21), and localized hemorrhages and pigmentation of ganglion cells in the pons. In the 2 patients with acute bulbar symptoms, the changes were largely limited to the medulla and pons (figs. 14, 21, and 22). Diplococci of varying size and shape, sometimes in short chains (figs. 15 and 23), were found in or adjacent to the lesions, and were proved absent in the areas remote from lesions in each of the 5 cases.

The findings in animals that died soon after injection were a variable degree of congestion of the vessels of the pia and brain, occasional slight general clouding of the meninges, localized infiltration of the pia over the anterior aspect of the midbrain, pons, and medulla, associated

with slightly or moderately turbid cerebrospinal fluid, small subpial hemorrhages, especially of the medulla and pons, more rarely of the cord and cerebral cortex, rarely a lesion at the point of inoculation, and a variable degree of hemorrhagic edema of the lungs and cloudy swelling of the viscera. Suppurative meningitis, when present, was almost always due to some organism (usually hemolytic streptococcus or staphyloccus) other than the green-producing streptococcus. Usually gross lesions were not found in the animals that died late. In some, marked increase in clear cerebrospinal fluid was found, simulating a true hydrocephalus.

The microscopic changes varied greatly according to the duration of the experiment, the size of the dose, and the virulence of the strain injected. Leukocytic and round-cell infiltration of the pia was most marked surrounding blood vessels in the sulci. Focal hemorrhages in the brain and medulla, often perivascular, associated with a variable degree of leukocytic and round-cell infiltration, and similar areas of infiltration unassociated with hemorrhage were the usual findings in the animals that succumbed or were anesthetized in from 1 to 5 days after injection. The organism was readily demonstrated in these lesions. As the duration of the experiment became longer, hemorrhages were less common, the leukocytes were displaced by round cells, and the diplococci became fewer and more irregular in size, large and small forms often occurring side by side and sometimes in the same chain. The proportion of leukocytes and round cells varied greatly according to the location of the lesion, the former often predominating in the pia and the latter in the perivascular spaces in the depths of the brain substance.

In the case of some strains, especially those isolated from emulsions of brain and cord or filtrates, round cells predominated almost from the beginning; in others, especially after several animal passages, the leukocytes persisted as the predominating cells for as high as 5 days after injection, particularly in animals that died. The round cells were chiefly lymphocytic, although some were large and resembled proliferated endothelial cells; still others had eccentrically placed nuclei and resembled plasma cells. Active phagocytosis of diplococci was uncommon in the animals that died, but often striking in the animals anesthetized when they were recovering from acute symptoms.

The cellular infiltration of the lesions induced by injection of the streptococcus from the different types of encephalitis was almost similar

(figs. 1, 3, 10, 11, 12, and 18). The differences in the symptoms observed were due in large part to the distribution of the lesions. Relatively marked lesions in the basal ganglions were found in all types of the disease, but, in addition, the lethargic and parkinsonian groups had extensive parenchymatous and perivascular infiltration in the subcortical region and in the region corresponding to the corpus striatum (fig. 3), the myoclonic group in the cortical region, the meningitis group in the meninges (fig. 11) and cortex, and those with bulbar symptoms, usually in the medulla and pons (fig. 18).

TABLE 4

SUMMARY OF FINDINGS ON MICROSCOPIC EXAMINATION OF SECTIONS OF THE CENTRAL NERVOUS SYSTEM

Source	Cases	Lesions of the Brain of Animals Injected								
		Intracerebrally with							Intravenously with Cultures of the Streptococcus	
		Material Containing the Streptococcus					Inert Filtrates, Salt Solution, and Broth			
		One to Five Days Before Necropsy			Six or More Days Before Necropsy					
		Number	Leukocytic and Round-Cell Infiltration	Characteristic Round-Cell Infiltration	Number	Characteristic Round-Cell Infiltration	Number	Characteristic Round-Cell Infiltration	Number	Characteristic Round-Cell Infiltration
Epidemic encephalitis	76	173	170	4	138	120	44	0	12	6
Encephalitis contacts	11	12	12	0	10	10				
Total	87	185	182	4	148	130	44	0	12	6
Miscellaneous	76	30	23	0	50	2	18	0	105	3
Normal controls 1922 and 1923	54	19	19	0	60	6				
Total	130	49	42	0	110	8	18	0	105	3

In the experiments of short duration, lesions of the choroid plexus were common, and consisted chiefly of leukocytic infiltration; in those of longer duration, they were less common, but occasionally there was marked perivascular round-cell infiltration, in which gram-positive diplococci were sometimes demonstrated.

The incidence and character of microscopic lesions following intracerebral and intravenous injection of material containing the streptococcus, inert filtrates, sterile broth, and sodium chloride solution are summarized in table 4. Sections of the brain were examined in 389 animals injected with material from 76 patients with encephalitis and

11 normal encephalitis contacts, and in 264 animals injected in a similar manner with material from 76 patients suffering from diseases other than those of the nervous system, and from 54 normal controls. The relative incidence of a variable degree of leukocytic and round-cell infiltration in the meninges or elsewhere following intracerebral inoculation was similar in these two groups. Thus in the encephalitis and encephalitis contact group, it occurred in 182 of 185 examined, and in the miscellaneous and normal control group in 42 of 49.

The relative incidence, however, of characteristic round-cell infiltration in experiments that ran for 6 days or more was far greater in the encephalitis and encephalitis contact group than in the miscellaneous and normal control group, 130 of 148 (87%) in the former, and only 8 of 110 (7%) in the latter. Similar results have been obtained in epidemic hiccup, spasmodic torticollis, and respiratory arhythmia. Characteristic lesions following intravenous injection of the streptococcus from encephalitis occurred in 6 of 12 animals examined, whereas following injection of streptococci from miscellaneous sources they were found in only 3 of 105. They were not found in any of 52 rabbits injected with inert filtrates, sterile broth, or sodium chloride solution. The characteristic round-cell infiltration which was found in 4 rabbits of 185 injected intracerebrally a short time before, and in 3 of the 105 injected intravenously with streptococci from miscellaneous sources, a total incidence of 2.5%, was no doubt of spontaneous origin. Moreover, it was not encountered in sections of the brain and medulla of 24 rabbits injected with filtrates nor in those of 29 uninjected rabbits anesthetized for cultural purposes.

The characteristic streptococcus was often isolated from the brains of animals that developed typical symptoms, even when the platings of the material from the throat showed a mixture of organisms. This was true even in instances in which the hemolytic streptococcus formed a large proportion of the colonies, provided the patient had not had a throat infection at the time. But if the patient had had an attack of acute tonsillitis a short time previously, suppurative meningitis due to the hemolytic streptococcus usually developed. Since certain strains had no invasive power, and others caused death from meningitis, I thought that certain strains of hemolytic streptococci might be of a proper grade of virulence to induce characteristic symptoms and lesions. Opportunity to test this hypothesis was presented in the following case of encephalitis.

A man, aged 58, was semicomatose from encephalitis which began 6 weeks previously, following an attack of tonsillitis. He recovered slowly. Cultures from the throat yielded a large number of hemolytic streptococci, and those from the clear spinal fluid, obtained on two occasions, a pure culture of the hemolytic streptococcus in glucose-brain broth, but no growth on blood-agar plates. All of 5 rabbits injected intracerebrally, 3 with the spinal fluid and 2 with the filtrate of nasopharyngeal washings, remained well. Four rabbits injected with varying doses of the cultures from the spinal fluid died of acute suppurative meningitis. One injected intravenously became ataxic and weak, and lost much in weight, but recovered. One injected intracerebrally with the sodium chloride solution suspension of the washings of the swab from the nasopharynx developed marked tremor, and paralysis of the muscles of respiration, of which it died on the 3rd day. Necropsy revealed numerous pial and subpial hemorrhages over the pons and medulla, and slightly turbid cerebrospinal fluid. Cultures yielded a pure growth of hemolytic streptococci, and sections revealed marked perivascular and parenchymatous infiltration by leukocytes and a few round cells, most marked in the pons and medulla, in which the streptococcus was readily demonstrable. Infiltration in the meninges was slight and limited almost entirely to the perivascular areas.

CONTROL EXPERIMENTS

In view of the fact that the "poliococcus" was demonstrated in the throats not only of persons who had poliomyelitis but also of normal persons, during the epidemic of poliomyelitis in 1921, and proved absent in 48 persons a year later when no cases occurred, I determined, during the spring of 1923, coincident with the occurrence of cases of encephalitis, to study the infecting power of the bacteria both from normal persons who were in contact with the disease and those who were not, and to compare these results with those obtained in cases of encephalitis.

The results following intracerebral inoculation of sodium chloride suspensions of throat swabs from 48 normal persons during the summer of 1922, all of whom were carriers of the "poliococcus" a year previously during the epidemic of poliomyelitis, and of 37 normal persons not in intimate contact with cases of encephalitis during the spring of 1923, are summarized as control experiments in table 1. Detailed findings in connection with the latter group, together with 11 encephalitis contacts, in some of whom the carrier state was demonstrable, are of particular interest.

During April and May, 1923, soon after an epidemic of mild influenza, when a number of cases of encephalitis occurred in Rochester and the surrounding country, intracerebral inoculations of suspensions

in sodium chloride solution of throat swabs from 40 normal persons were made in the usual manner in 88 rabbits. Sixty remained free from symptoms. Of the remaining 28, 21 developed a variable degree of ataxia; 14, turning of the head; 14, hyperpnea; 8, paralysis; 7, tremors; 5, nystagmus; 4, spasms of muscles; 2, tic-like movements of the head, and 2, lethargy. Thirteen of the 21 with ataxia, and 9 of the 14 with turning of the head and hyperpnea, respectively, died within 5 days after injection, with evidence of meningitis. Altogether, 38 died in from 1 to 28 days after inoculation. A green-producing streptococcus, the cause of death, was isolated from the brain in 22. In 8, death was due to the bacillus of rabbit septicemia; in 2, to the staphylococcus, and in 1, to the hemolytic streptococcus; in the other 5 the cause of death was not definitely established. Marked congestion of the vessels of the meninges, with moderate turbidity of the spinal and ventricular fluids, was the chief finding at necropsy in the animals that died within 5 days after inoculation; gross lesions were not found in those that were anesthetized or that died a long time after. In one, an abscess had formed at the point of inoculation.

Sections of different parts of the brain and medulla of 76 animals, representing 40 persons, were examined microscopically. Leukocytic infiltration of the meninges and the ventricles usually with slight infiltration of surrounding structures dominated the picture in the animals that died soon (1 to 5 days) after inoculation; sufficient time (6 to 32 days) had elapsed for the development of typical lesions, such as perivascular round-cell infiltration, in 45 animals. Characteristic lesions, chiefly in the deep layers of the cerebrum and in the basal nuclei, were found in 13 of these, representing 10 persons. Ten of these 13 rabbits had been injected with material from 7 normal persons, members of 3 families, in each of which a case of encephalitis had developed. Typical lesions were found in 1 each of 2 rabbits injected with material from husband and wife, respectively. The husband was being treated for chronic rhinitis, the wife for chronic sinusitis. The remaining rabbit, which had typical lesions, had been injected with material from the nasopharynx of a person suffering from chronic otitis media. The remaining 32 animals that lived long enough for typical lesions to develop, but were free from lesions, had been injected with material from the throats of 17 normal persons who had not been in intimate contact with patients, and who were free from sinusitis or abnormal throat or ear conditions. Lesions were not found in sections of the

brains of 14 rabbits of the series that were chloroformed as controls. Cultures from the blood and brain were sterile in all.

Of the 22 strains of green-producing streptococci isolated from the brains of animals that succumbed, 11 produced diffuse turbidity in glucose broth or glucose-brain broth, suitable for agglutinating purposes. Seven of these were agglutinated by my encephalitis and poliomyelitis serums, but not by pneumococcus serums (types I, II, and III), nor by normal horse serum. Four of these strains had been obtained from the throats of normal persons in contact with patients who had encephalitis, and produced typical microscopic lesions in animals.

The strains from normal persons, which were agglutinated specifically by the encephalitis and poliomyelitis serums, resembled, morphologically and culturally, those isolated from patients with encephalitis. The streptococcus was demonstrated in the lesions and proved absent in normal tissues. According to the microscopic lesions, and the agglutination experiments, 13 of 40 normal persons examined during the prevalence of encephalitis in the community must be regarded as carriers of the encephalitis streptococcus; and what is more significant, 10 of these resided in the households of 3 families in each of which a case of encephalitis had developed, and 3 were suffering from low grade infections of the nasopharyngeal mucous membrane or the accessory sinuses.

In view of the high incidence of positive microscopic changes and of the specific agglutinating reaction of the streptococcus from persons in more or less intimate contact with cases of encephalitis, I divided the animals into 2 groups according to whether the material injected was from the throat of persons who were or who were not in intimate contact with the disease. As indicated in table 1, the incidence of disease and the mortality rate in the former group was decidedly higher than in the latter. Thus, of the 30 rabbits injected with material from 11 normal persons in contact with encephalitis, 4 (13%) developed spasms of muscles, 3 nystagmus, one tic-like movements, 14 (47%) ataxia, 10 (33%) turning of the head, 5 (17%) tremors, 7 hyperpnea, 4 paralysis, and 2 lethargy; whereas of the 58 rabbits injected with material from 29 normal persons not in intimate contact with this disease, none developed spasms of muscles, or lethargy; 2 had nystagmus, 2 tic-like movements, 9 (14%) ataxia, 5 (8%) turning of the head, 2 tremors, 7 hyperpnea, and 4 paralysis. The mortality in the former group, attributable to the material injected, was 40%; in the latter, 17%.

SUMMARY OF RESULTS IN THE THREE FAMILIES IN WHICH THE CARRIER STATE WAS DEMONSTRATED

Family 1.—In a family of 5, a boy, aged 9 years, developed symptoms of myoclonic encephalitis without apparent cause. On March 13, 1923, the 4th day of his illness, 2 rabbits were injected intracerebrally with the salt suspension of the nasopharyngeal swab. Both developed myoclonic twitchings and other symptoms of encephalitis. One died in 3 days, the other in 10.

The filtrate of the emulsion of the brain of the rabbit that died in 3 days was injected intracerebrally into 2 rabbits. One remained free from symptoms; the other developed severe spasms of muscles, including spasms of the diaphragm, 6 hours after injection. It was placed under light ether anesthesia, and the diaphragm exposed through a middle line abdominal incision. Spasms of the diaphragm were seen and palpated. The filtrate of the emulsion of the brain of the rabbit that died 10 days after injection, which proved sterile in cultures, was without effect in 2 rabbits injected.

Two rabbits were injected in the usual way with filtrates of the nasopharyngeal washings of the patient. Fifteen minutes after injection both developed tremor and twitching of the muscles, which lasted for half an hour. They remained well subsequently, and sections of the brain revealed no lesions. Two rabbits injected with the spinal fluid from the patient remained well, and were free from lesions.

The patient recovered promptly following administration of the encephalitis serum; and coincident with his recovery, the streptococcus disappeared from his throat, 2 rabbits injected with material from a throat swab remaining well. Three weeks later, the patient's symptoms returned, but again disappeared promptly after cautious administration of the serum. May 8, four weeks after this recurrence, the organism was again demonstrable in his throat, and 2 rabbits injected developed encephalitis.

The effect of intracerebral inoculation of a suspension in sodium chloride solution of the nasopharyngeal swabs of the father, mother, one older brother, and a younger sister, was studied at the time of the patient's attack, March 13, and again on May 8. The characteristic streptococcus was demonstrated at the time of the attack in the throat of the child's brother, who had slept with the patient, and of his mother, who took care of him, but was not demonstrable two months later. The results in the father and sister, who were in less intimate contact with the patient, were negative at the time of the attack, but one month later positive results were obtained in both, and typical microscopic lesions were found in the brain of inoculated rabbits.

Family 2.—The youngest child, aged 3, developed lethargic encephalitis following a cold which had passed through the household. The family lived on a farm in a region where other cases of encephalitis had developed, but the patient had not been exposed to the disease.

Four rabbits injected with material from the patient at the time of the attack died from encephalitis in from 1 to 6 days. The mother and brother, both of whom had been in intimate contact with the patient, harbored the encephalitis streptococcus in their throats, 3 of 4 rabbits yielding positive results. The throats of the father and another brother, both in less intimate contact with the patient, were free from the streptococcus, 4 rabbits injected remaining well and free from microscopic changes in the brain.

Family 3.—An old man, in a family of 4 persons, developed marked lethargy during an attack of bronchopneumonia, from which he ultimately recovered. At the time of his attack the results were positive in 1 each of 2 rabbits injected.

respectively, with material from the patient and from his daughter, who took care of him; whereas 2 rabbits injected with material from the patient's wife, who had been ill and not in contact with him, remained free from symptoms and lesions.

Besides the cases summarized in tables 1 and 4, negative results were obtained following the intracerebral injection of 66 rabbits with the streptococcus from 24 additional sources. In these experiments, the strain was from the nasopharynx of 6 patients with residual symptoms of encephalitis, and in 3 patients that had recovered from encephalitis a long time before. In 1 it concerned a case of familial or essential tremor, in 2 of brain tumor, in 1 of tuberculous meningitis, and in 1 of cerebral syphilis, which were thought to be encephalitis at the time of study. One strain was from the throat of a patient with herpes zoster, 1 with cerebellar tumor associated with persistent hiccup, 2 from the cervix of patients with endocervicitis, and 6 from the nasopharynx of normal persons during March, 1921. Symptoms and lesions were negative following injections of highly virulent strains of types I and II pneumococci.

SUMMARY OF EXPERIMENTS ON INTRAVENOUS INJECTION

The freshly isolated strain of the streptococcus from 18 patients with encephalitis, nearly all acute cases, was injected intravenously into 93 rabbits. Forty-one died, a mortality of 44%. Of the 93, spasms of the diaphragm developed in 5, and of abdominal muscles in 11, ataxia in 16, tremors in 28, hyperpnea in 8, paralysis in 20, and lethargy in 11. Cultures were made in 56 instances. In 22 of these, the strain was isolated from the brain, and in 9 from the blood. Sections of the brain were made in 12, 6 of which revealed characteristic lesions.

The incidence of encephalitic symptoms, of the isolation of the streptococcus from the brain, and of lesions, in this series is far greater than following intravenous injection of streptococci in like dosage from similar sources, but from diseases other than encephalitis. Thus, of 105 rabbits, symptoms of encephalitis developed in only 1, which had been injected with a green-producing streptococcus from the apex of a devitalized tooth in a patient with arthritis, and the streptococcus was isolated from the brain. The blood was sterile; sections of the brain revealed localized areas of leukocytic and round-cell infiltration. In 3 of the 105, perivascular round-cell infiltration of spontaneous origin was found. Cultures were made of the brain and blood in all. In only 12 was a green-producing streptococcus isolated.

SUMMARY OF EXPERIMENTS IN MICE

Tests consisting of intraperitoneal injections in mice of the saline suspension of the material from the nasopharynx, and more often of cultures of the streptococcus, soon revealed the fact that the virulence of the organism was low; it rarely produced death from exudative peritonitis. It was noted, however, that some of the mice that died without gross peritonitis had symptoms suggesting encephalitis, and in these the streptococcus was isolated from the brain. This was contrary to the results following inoculation of other strains. Because of this finding, sections were made of the brains of some of the mice that died.

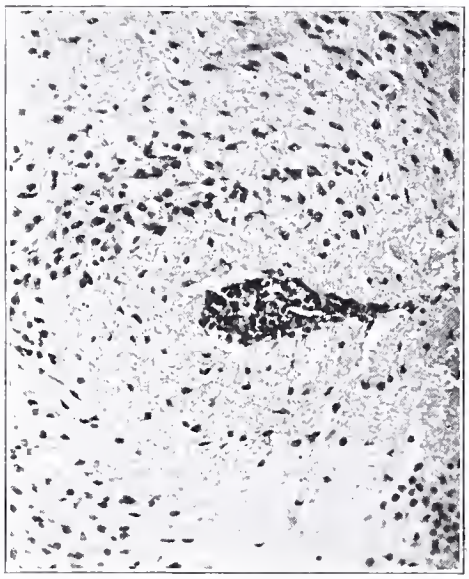

Fig. 24.—Leukocytic and round-cell infiltration in the brain of mouse 732, 4 days following intraperitoneal injection of strain 4226 (case 7) after one rabbit passage. Hematoxylin and eosin (× 120).

A study of sections, together with the results of cultures and gross findings, was made in 36 mice, representing the streptococci from 8 cases of acute encephalitis. A variable degree of leukocytic and round-cell infiltration in the brain in which the streptococcus was demonstrated was found in 24. In those in which there were lesions, the duration of the experiment was 1 day in one; 2, 3, and 4 days in two each; 5 days in five; 7 days in six; and 10 to 16 days in six; whereas in those without lesions, it was 1 day in one, 2 days in six, and 15 days in one. Slight peritonitis was present in only 7, all in experiments of short duration. Cultures from the brain yielded the streptococcus in 32 mice, those from the blood in 23.

In some instances, the microscopic lesions were extreme, a large part of the brain showing perivascular and other areas of hemorrhage, and cellular infiltration. The meninges and perivascular spaces in sulci often were markedly infiltrated; in others, the lesions were slight and limited almost entirely to the midbrain region. The character and proportion of the infiltrated cells varied greatly with different strains and with the duration of the experiment (figs. 24 and 25). Leukocytes were present almost exclusively in the experiments of short duration; as the duration became longer, a larger proportion of round cells were found, but in no instance did they entirely displace the leukocytes. In some experiments of equal duration with the same strain, leukocytic infiltration was marked in the brain in mice, whereas round-cell infiltration predominated in the brain in rabbits. Large numbers of diplococci were found in the lesions in experiments of short duration, and relatively few in those of longer duration (fig. 26).

SUMMARY OF EXPERIMENTS WITH SPINAL FLUID AND EMULSIONS OF THE BRAIN

One or more specimens of spinal fluid from 33 cases were cultivated and injected. The duration of the disease at the time of the spinal puncture ranged from 4 days to 5 months. The characteristic streptococcus was isolated in 7 cases and a hemolytic streptococcus in 2 specimens from 1 case. Three of the 7 were of the meningitis group, and the spinal fluid was turbid, but in only 1 (case 7) was the organism present in large numbers. In the others in which the streptococcus was isolated, the fluid was clear and contained the usual number of cells.

At first intracerebral inoculations were made, but later, owing to the results of Loewe and Strauss, subdural injections were given. Sixty-seven rabbits were injected with spinal fluid, exclusive of strain 4226 of the meningitis group. Only 2 of these rabbits developed symptoms, consisting of convulsions in one immediately after injection, and of meningitis in the other two days after injection. The latter was found to be due to accidental infection by gram-negative bacilli. Cultures of the brain and blood of 30 animals anesthetized for examination in from 4 to 30 days after inoculation remained sterile in all instances, and sections of the brain of 24 were free from lesions.

Emulsions of the brain from the patients who died were injected into 29 rabbits. Four developed symptoms; in 2 these were due to accidental infection with gram-negative bacilli; in 2 the characteristic streptococcus was isolated. The cultures were sterile in 20 that did not

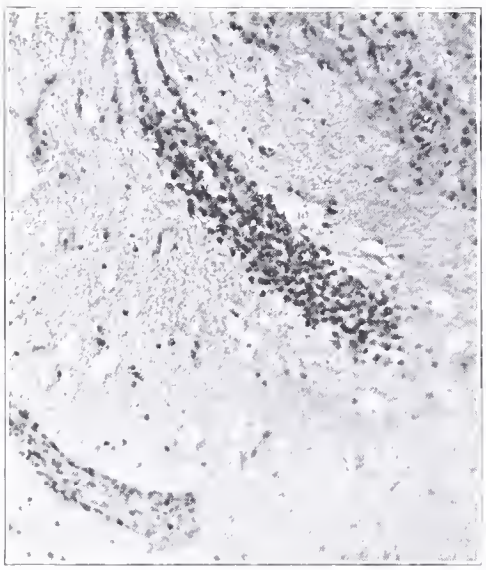

Fig. 25.—Perivascular round-cell infiltration in brain of a mouse 16 days after intra peritoneal injection of a streptococcus from encephalitis. Hematoxylin and eosin (× 120).

Fig. 26.—Diplococci in the lesions of the brain shown in figs. 24 and 25.

have symptoms. Sections were made in 12 animals, and characteristic lesions were found only in 2 that developed symptoms and from which the streptococcus was isolated in cultures.

SUMMARY OF EXPERIMENTS WITH FILTRATES

Eighty-three filtrates of material from 41 patients were injected intracerebrally into 157 rabbits (table 5). Twenty-seven of these were prepared from the nasopharyngeal washings of 26 patients, 34 from emulsions of the brain of positive rabbits injected with material from 22 patients, 5 from emulsions of the brain from 5 patients that died, and 17 from pure cultures of the streptococcus from 9 patients. In 21

TABLE 5

SUMMARY OF RESULTS IN RABBITS INJECTED WITH FILTRATES

Number of Patients	Filtrates		Animals Injected	Animals Developing Symptoms of Encephalitis	Microscopic Findings in Brain		Animals Cultured	Characteristic Streptococcus Isolated from	
	Source	Number			Animals Examined	Animals with Characteristic Lesions		Brain	Blood
26	Nasopharynx	27	54	17	11	8	32	9	2
5	Emulsion of brain (human)	5	27	0	9	0	14	0	0
9	Cultures	17	27	5	6	3	18	2	0
22	Emulsion of brain of positive rabbits	34	49	16	14	11	26	13	3
41	Total	83	157	38	40	22	90	24	5

instances, filtrates from more than one of these sources were injected. Direct cultures yielded the characteristic streptococcus, always in pure form, in 10 of the 27 filtrates prepared from nasopharyngeal washings; in 12 of the 34 from the emulsion of the brain of positive rabbits; in 4 of the 5 from the emulsions of the brain in fatal cases; and in 6 cases of the 17 from cultures. Thus, 33 of the 83 filtrates yielded this organism, always in pure form, by direct cultures.

The ages of the 41 patients ranged from 1½ to 62 years, the duration of the disease from 4 days to 3½ years. In 7, the symptoms were acute and were present for 10 days or less; in 5, the duration of symptoms was between 10 and 30 days; in the others, from 1 month to 3½ years.

Different types of the disease were represented; 7 were predominantly parkinsonian, 4 lethargic, 3 myoclonic, 2 meningitic, and the

others were of the mixed or unclassified type. Positive results were obtained with filtrates from each group, and nearly always in the early stages of the disease. In a few acute cases, in which tremor was a predominant symptom, the freshly prepared filtrate of nasopharyngeal washings was toxic immediately after injection, tremor and twitchings of muscles developing in from 15 to 30 minutes after injection. The results, arranged according to the source of the material from which the filtrate was prepared, are summarized in table 5.

Seventeen of 54 rabbits injected with 27 filtrates of material from the nasopharynx of 26 patients developed symptoms of encephalitis. Eight of 11 rabbits examined had characteristic lesions of the brain, and in 6 the organisms were demonstrated in the lesions. Nine of 32 from which cultures were taken yielded the characteristic streptococcus in the brain, and 2 in the blood. None of 27 rabbits injected with 5 filtrates of the emulsion of the brain of 5 patients that died developed symptoms; no lesions were found in the brain of 9 of these rabbits in which sections were made, and the 14 from which cultures were taken were negative. Five of 27 rabbits injected with 17 filtrates, obtained from 9 patients, developed characteristic symptoms, 3 of 6 revealed characteristic lesions, and cultures from the brain were positive in 2 of 18.

Of the 157 rabbits injected with filtrates, a total of 38 developed symptoms of encephalitis, 22 of 40 were proved to have characteristic microscopic lesions, and in 24 the characteristic streptococcus was isolated. Cultures were made in 90 of the 157 rabbits. The 24 rabbits yielding positive results died, or were chloroformed for examination, during the early stages of the disease. In those in which cultures were made late, and in those that remained free from symptoms, 66 in all, the streptococcus was not isolated in a single instance. Characteristic results followed injection of the streptococcus isolated from filtrates directly, from filtrates of emulsions of the brain of patients that died, and from positive rabbits injected with filtrates, in some instances representing strains after many rapidly made subcultures. Positive results usually followed injection of filtrates that yielded the streptococcus in cultures, but not in all instances. This is in keeping with the fact that not infrequently rabbits injected with diluted cultures, suspensions of nasopharyngeal or tonsillar material, and with emulsions of the brain in fatal cases in which the streptococcus was isolated, remained free from symptoms, the result, no doubt, of inadequate dosage. Because

of the small number of bacteria in the filtrate, the symptoms, while similar to those following injection of the corresponding diluted culture, were delayed in onset, and usually less severe but more characteristic, and the mortality was correspondingly lower. Eighteen of the 22 rabbits that revealed characteristic lesions had symptoms during life; in the others, no symptoms had developed.

In order further to verify the results, emulsions of the brain of rabbits that remained free from symptoms after injection with material which it was thought might contain a filtrable virus were repeatedly injected into rabbits through one or more passages, always with negative results.

CHARACTERISTICS OF THE STREPTOCOCCUS

The streptococci isolated from the nasopharynx, foci of infection, and, in fatal cases, from the brain, were culturally and morphologically much alike, and produced in animals symptoms and lesions characteristic of the acute and chronic forms of the different types of the disease. The organism usually produced small, dry, slightly elevated, non-adherent colonies on blood-agar plate, surrounded by a narrow greenish or brownish zone on transmitted light, but occasionally they were flat, spreading, and umbilicated; in some instances, it produced indifferent colonies; in others, a narrow zone of partial hemolysis immediately surrounded the greenish zone. The colonies were usually not sufficiently characteristic to distinguish them from green-producing streptococci normally present in the throat. The number of green-producing colonies, often in nearly pure culture, on blood agar inoculated with material from nasopharyngeal swabs of patients, especially those with acute symptoms, was in general much larger than on those inoculated with material from normal patients. The size of the colonies, and the amount of green production usually increased as virulence became greater through rapid animal passage. Strains that had lost the power to produce green on blood agar often acquired it again when grown in glucose-brain broth, or when passed through animals. In glucose-brain broth and glucose broth, the streptococcus usually produced diffuse turbidity, which tended to become granular and to precipitate after from 48 to 72 hours' incubation. In some instances, this occurred within 12 hours after abundant growth. If the inoculum was small, as in filtrates, it was necessary to drive off the oxygen by boiling and cooling to 37 C. immediately before use, in order to facilitate growth. Warming the medium, especially if fresh, before inoculating, was

sometimes sufficient to produce growth, which began in the bottom of the tall tubes of glucose-brain broth, and gradually extended to the top. In young cultures in glucose-brain broth and blood agar, the organism was a gram-positive, nonencapsulated, sometimes slightly lanceolate diplococcus, occurring singly and in short chains, and varying considerably in size and shape. The larger forms approximated the size of pneumococci (fig. 4 *a*); the smaller ones were from one-eighth to one-half as large (fig. 4 *b*). In old anaerobic cultures, especially in meat infusion and in Noguchi's medium, the organism was very much smaller (fig. 4 *b*), and often extremely pleomorphic, as it often is in the experimental lesions (fig. 13), in lesions in the midbrain in man (fig. 15), and in smears from nasopharyngeal swabs. It is rarely filtrable in young glucose-brain-broth cultures, but commonly passes through bacterial filters when grown under conditions which induce extremely small forms, the growth from the filtrate, as in transfers from cultures, being characteristic of the medium inoculated.

Extremely large as well as small coccus forms were frequently found in smears of old anaerobic cultures, free, and also in chains of undoubted streptococci. Direct platings or platings of glucose-brain-broth cultures of these not infrequently yielded staphylococcus besides the streptococcus colonies. This occurred in some instances when the organism inoculated was from single colonies of pure cultures of the streptococcus from the throat or brain of patients, from the brain of inoculated animals, and from filtrates of these.

The fermentative power was tested in 47 strains. All produced acid in glucose, 22 in lactose, 38 in maltose, 31 in saccharose, 21 in raffinose, 10 in salicin, 13 in inulin, and none in mannite.

The most characteristic features of the streptococcus are the peculiar localizing power and the immunologic condition. Although of low general virulence, it has well marked and specific antigenic properties (table 6). The technic of immunization was similar to that used in connection with strain 4226. Two ulcer and 2 encephalitis strains were used.

AGGLUTINATION EXPERIMENTS

The standard technic was employed in the agglutination experiments. The antigen consisted of either an 18 to 24-hour culture of the streptococcus in glucose-brain broth, or a sodium chloride solution suspension about twice the density of the broth culture made from dense glycerol-sodium chloride solution suspensions of freshly isolated strains grown

in glucose broth. Two-tenths c c. of the antigen was added to an equal amount of the serum dilutions, thoroughly mixed, placed in the incubator for one and one-half hours, and in the ice chest over night; a preliminary reading was then made, and a second reading after heating to 55 C. for one and one-half hours. The results recorded in tables 7 and 9 were those of the second reading. The figures indicate the degree of agglutination, 0 representing no agglutination; 1, slight; 2, moderate; 3, marked, and 4, complete.

The anti-encephalitis and antipoliomyelitis serums were prepared by repeated injection of horses, over a long period, with increasing amounts of the respective freshly isolated strains preserved in dense glycerol-sodium chloride solution suspensions. The encephalitis serum from

TABLE 6

SPECIFICITY OF ANTIGENIC PPROPERTIES OF THE STREPTOCOCCUS FROM ENCEPHALITIS

Test Strains	Rabbits		Animals Injected	Localization in	
				Stomach	Brain
Ulcer	Normal		28	23 (82%)	0
	Immunized with	Ulcer vaccine	23	4 (17%)	0
		Encephalitis vaccine	7	6 (86%)	0
Encephalitis	Normal		12	2 (17%)	9 (75%)
	Immunized with	Ulcer vaccine	6	0	4 (67%)
		Encephalitis vaccine	6	0	0

horse 18 was prepared with 1 strain (strain 3900); the other encephalitis serum (serum 234) and the antipoliomyelitis serum (serum 212) were prepared by injecting varying amounts of 3 and 11 strains, respectively. The antipneumococcus serums were obtained from the New York State Department of Health.

Representative results in 18 cases of encephalitis, in 3 cases of poliomyelitis, and in 6 control strains are given in table 7. All of the strains were from positive animals injected with material from the nasopharynx. The strains varied markedly in susceptibility to agglutination. A few were agglutinated by all serums in low dilution, others markedly by the encephalitis and poliomyelitis serums and not at all by type pneumococcus and normal serums, whereas others were not agglutinated by any serum.

TABLE 7

AGGLUTINATION EXPERIMENTS WITH SERUM OF HORSES IMMUNIZED WITH THE STREPTOCOCCUS FROM ENCEPHALITIS AND POLIOMYELITIS RESPECTIVELY

Strain	Source of Streptococcus	Diagnosis	Duration of Illness	Serum Dilutions: Anti-encephalitis H 18				Anti-encephalitis 234				Antipoliomyelitis 212				Anti-pneumococcus I				Anti-pneumococcus II				Normal Horse				Sodium Chlorid Solution
				1:20	1:100	1:200	1:1000	1:20	1:100	1:200	1:1000	1:20	1:100	1:200	1:1000	1:20	1:100	1:200	1:1000	1:20	1:100	1:200	1:1000	1:20	1:100	1:200	1:1000	
4642	Filtrate, pons and medulla	Acute encephalitis	8 days	4	2	2	2	4	2	2	0	4	3	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0
4688	Nasopharynx	Acute lethargic encephalitis	3 weeks	3	3	2	0	3	2	2	0	3	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4702	Tonsil pus	Encephalitis, striate syndrome	1 year	4	2	0	0	4	2	0	0	4	2	2	0	0		0	0	0	0	0	0	0	0	0	0	0
4705	Nasopharynx	Beginning parkinsonian syndrome	14 months	4	2	1	0	4	4	2	0	4	3	0	0	2	0	0	0	0	0	0	0	2	0	0	0	0
4711	Nasopharynx	Acute encephalitis	1 week	3	4	2	0	4	4	2	0	4	3	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
4713	Infected teeth	Acute encephalitis	2 weeks	3	2	0	0	3	2	0	0	3	0	0	0	...	...	...	...	...	...	...	...	2	0	0	0	0
4714	Nasopharynx	Encephalitis, striate syndrome	2 years	3	2	2	0	3	3	2	0	3	3	0	0	...	...	...	...	...	...	...	...	0	0	0	0	0
4716	Spinal fluid	Acute lethargic encephalitis	1 month	3	2	2	0	3	2	0	0	3	3	2	...	...	...	...	...	...	...	...	...	0	0	0	0	0
4722	Nasopharynx	Acute meningo-encephalitis	2 weeks	4	3	3	2	4	3	3	2	4	3	3	2	...	...	...	...	...	...	...	...	3	2	2	2	2
4724	Nasopharynx	Acute myoclonic encephalitis	5 days	2	2	3	3	2	2	4	2	4	3	3	2	3	0	0	0	2	0	0	0	0	0	0	0	0
4730	Apex, infected tooth	Parkinsonian syndrome	4½ years	3	2	0	0	3	3	3	2	4	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4732	Infected teeth	Acute encephalitis	7 weeks	4	3	3	2	4	3	3	2	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4736	Nasopharynx	Parkinsonian syndrome	3 years	4	2	2	0	4	2	0	0	4	4	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0
4738	Nasopharynx	Parkinsonian syndrome	2½ years	2	0	0	0	2	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4739	Nasopharynx	Acute myoclonic encephalitis	1 week	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4740	Nasopharynx	Acute lethargic encephalitis	2 weeks	3	0	0	0	2	0	0	0	3	3	2	1	2	0	0	0	2	0	0	0	3	0	0	0	0
4742	Nasopharynx	Acute bulbar encephalitis	10 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4742	Filtrate, medulla	Acute bulbar encephalitis	10 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4659	Medulla	Acute sporadic poliomyelitis	1 week	4	4	4	3	4	4	4	3	4	4	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0
4709	Tonsil	Chronic poliomyelitis	6 months	4	2	0	0	4	2	0	0	4	4	3	0	...	...	...	...	...	...	...	...	2	0	0	0	0
4720	Nasopharynx	Spasmodic torticollis and bulbar palsy	6 months	3	2	0	0	3	2	2	0	3	2	0	0	...	...	...	...	...	...	...	...	2	0	0	0	0
284	Nasopharynx	Normal person	.....	3	2	2	2	3	2	2	2	3	2	2	2	...	...	...	...	...	...	...	...	3	2	2	2	2
1176	Tonsil	Normal person	.....	3	3	3	0	3	3	3	0	3	3	3	0	...	...	...	...	...	...	...	...	3	3	3	0	0
4682	Ulcer in dog	Ulcer of stomach in a dog	.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4684	Tonsil	Acute recurring ulcer of stomach	1 month	2	2	0	0	0	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0	0
4725	Nasopharynx	Normal person (brother, Case 4724)	.....	0	0	0	0	0	0	0	0	4	4	4	0	...	...	...	...	...	...	...	...	0	0	0	0	0
Mixture of five ulcer strains of streptococci				2	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0

With few exceptions, the agglutinating power of the encephalitis and poliomyelitis serums over the respective strains ran parallel. Thus, of 20 experiments in the 18 cases of encephalitis, specific agglutination occurred in both encephalitis serums in 16 instances, representing 16 cases, and in the poliomyelitis serum in 16; in only 2 cases were the results at variance in these serums. All 3 poliomyelitis strains and none of the control strains were agglutinated specifically by these serums. One of the latter, from the brother of the patient in case 9 (acute myoclonic encephalitis), with whom he had been in close contact, was agglutinated by the poliomyelitis but not by the encephalitis hyperimmune serum. The agglutinating titers of the strain from the nasopharynx and medulla respectively in two cases (cases 8 and 9) were

TABLE 8

SPECIFIC AGGLUTINATION BY THE ENCEPHALITIS AND POLIOMYELITIS HYPERIMMUNE SERUMS, GREEN-PRODUCING STREPTOCOCCI FROM INFECTION ATRIA OF PATIENTS WITH ENCEPHALITIS AND THOSE WITH MISCELLANEOUS DISEASES, AND OF NORMAL CONTROLS

Number of Cases	Source of Strains	Cases Studied	Experiments	Specific Agglutination in	
				Number of Cases	Percentage of Experiments
67	Epidemic encephalitis:				
	As isolated	26	68	11	51
	After animal passage, and from brain and cord	65	261	51	71
	After long cultivation	18	27	8	33.3
88	Miscellaneous diseases:				
	As isolated	46	62	6	10
	After animal passage	59	71	10	13
48	Normal controls:				
	As isolated	24	27	0	0
	After animal passage	32	37	2	5

almost identical, one each being agglutinated markedly, the other not at all. Specific agglutination occurred in strains that were isolated from various foci of infection in both acute and chronic forms of various types of the disease, and from the medulla and spinal fluid in one instance each of acute encephalitis.

The results of agglutination experiments with the streptococcus from encephalitis, miscellaneous diseases, and normal controls are summarized in table 8. When agglutination of a particular strain was more marked, and when it occurred in higher dilutions in the encephalitis and poliomyelitis hyperimmune serums than in the type pneumococcus serums, immune streptococcus serum from influenza, and normal horse serum, it was considered specific agglutination.

The results were grouped according to whether the strain subjected to the agglutination tests was cultivated directly from the infection atria or after one or two animal passages. It will be noted that specific agglutination of the streptococcus as isolated, usually in the primary glucose-brain-broth culture, occurred in a smaller percentage of instances than after one or two animal passages. Thus, in the case of the encephalitis strains, specific agglutination occurred as isolated in 51% of 68 experiments, representing 11 of 26 cases, whereas after animal passage, specific agglutination occurred in 71% of 261 experiments, representing 51 of 65 cases. The tendency of these strains to lose their specificity on artificial mediums is illustrated by the fact that specific agglutination by these serums occurred in only 33.3% of 27 experiments, representing 8 of 18 cases.

The results are in sharp contrast to those with streptococci from similar sources in miscellaneous diseases and normal controls, the highest incidence of specific agglutination being 13% of 71 experiments, representing 10 of 59 cases in the miscellaneous diseases, and 5% of 37 experiments, representing 2 of 32 normal controls.

In the early part of the work, routine agglutination experiments with the serum of patients were performed with the strains isolated, which were thought to be specific. The results were inconsistent because nearly all patients studied had chronic forms of the disease. It was noted, however, that the agglutinating power increased markedly in from 24 to 48 hours after injection of immune encephalitis or poliomyelitis serum. During the increased prevalence of encephalitis last spring, acute cases were studied, in which it was proved that specific agglutinins were present in the serum in demonstrable form. Representative results are given in table 9 of experiments on the agglutinating power of the serum of patients with encephalitis, over encephalitis, poliomyelitis, and control strains.

The serum from the various patients differed greatly in agglutinating power. In general, agglutination was more marked and occurred with a larger number of strains in the serums from acute cases (serums 4732, 4739, and 4742) than in the serums of patients with mild and chronic forms of the disease (serums 4735 and 4736). The agglutinating power increased markedly although temporarily in two cases (cases 4735 and 4740) following injection of the immune horse serum. The agglutination of the serum of the patient in case 4735, March 21, was relatively slight. Treatment with the immune horse serum was begun the next day; 4 daily doses of 20 c c. each were given. Marked increase

TABLE 9

AGGLUTINATING POWER OF THE SERUM OF PATIENTS WITH ENCEPHALITIS

Serums	Diagnosis	Duration of Symptoms	Serum		Strains of Streptococci from									
					Encephalitis								Poliomyelitis, 570 (Mixture of 5 Strains)	Ulcer, Mixture of 3 Strains
			Date Obtained	Dilutions	3900	3900.13*	4730².5*	4732².2*	4740².3*	4742	571 (Mixture of 13 Strains)	574 (Mixture of 4 Strains)		
4732	Acute encephalitis	2 weeks	3/24	1:4	4	3	3	5	4	4	4	4	4	2
				1:20	4	0	3	3	4	4	4	4	4	0
				1:40	3	0	3	2	3	4	4	4	4	0
				1:200	2	0	2	1	2	2	3	0	0	0
4735 (Case 6)	Myoclonic encephalitis	9 weeks	3/21	1:4	2	0	3	2	2	2	3	0	0	0
				1:20	0	0	2	0	2	0	0	0	0	0
				1:40	0	0	2	0	2	0	0	0	0	0
				1:200	0	0	0	0	0	0	0	0	0	0
			3/23	1:4	4	3	3	3	2	3	3	3	3	2
				1:20	4	2	2	2	2	3	3	2	3	0
				1:40	3	0	2	1	2	3	3	2	3	0
				1:200	2	0	1	0	2	0	2	0	1	0
			3/30	1:4	...	...	2	...	...	2	2	2	0	2
				1:20	...	...	0	...	...	2	0	0	0	0
				1:40	...	...	0	...	...	2	0	0	0	0
				1:200	...	...	0	...	...	0	0	0	0	0
4736	Parkinsonian syndrome following encephaltis	3 years	3/30	1:4	2	0	0	0	2	0	0	3	3	2
				1:20	0	0	0	0	2	0	0	2	0	0
				1:40	0	0	0	0	2	0	0	2	0	0
				1:200	0	0	0	0	2	0	0	0	0	0
4739	Acute myoclonic encephalitis	2 weeks	3/30	1:4	2	0	2	2	2	2	0	2	2	2
				1:20	2	0	2	0	2	2	0	0	2	0
				1:40	2	0	0	0	3	0	0	0	2	0
				1:200	0	0	0	0	1	0	0	0	1	0
4740	Acute lethargic encephalitis	2 weeks	3/23	1:4	3	0	2	3	3	3	2	0	3	0
				1:20	2	0	0	3	3	3	2	0	2	0
				1:40	0	0	0	0	2	2	1	0	0	0
				1:200	0	0	0	0	0	2	0	0	0	0
			3/24	1:4	4	0	3	3	3	3	3	3	3	2
				1:20	3	0	0	3	3	3	3	3	3	0
				1:40	2	0	0	2	3	3	2	2	2	0
				1:200	0	0	0	0	3	2	2	0	1	0
			4/ 6	1:4	2	0	3	2	3	3	2	0	2	0
				1:20	0	0	0	0	3	3	2	0	0	0
				1:40	0	0	0	0	2	2	0	0	0	0
				1:200	0	0	0	0	0	1	0	0	0	0
4742 (Case 9)	Acute bulbar encephalitis	10 days	3/31	1:4	0	0	2	3	3	2	0	2	2	2
				1:20	0	0	1	2	3	2	0	0	0	0
				1:40	0	0	0	2	3	2	0	0	0	0
				1:200	0	0	0	0	2	0	0	0	0	0
A	Normal control	.....	3/20	1:4	0	0	0	0	0	0	0	0	0	2
				1:20	0	0	0	0	0	0	0	0	0	0
				1:40	0	0	0	0	0	0	0	0	0	0
				1:200	0	0	0	0	0	0	0	0	0	0
B	Normal control	.....	3/20	1:4	0	0	0	0	2	0	0	0	0	0
				1:20	0	0	0	0	1	0	0	0	0	0
				1:40	0	0	0	0	0	0	0	0	0	0
				1:200	0	0	0	0	0	0	0	0	0	0
Sodium chloride solution (control)					0	0	0	0	0	0	0	0	0	0

* The exponent represents the animal passage; the figure following the period, the number of subcultures.

in agglutinating power was noted March 23, but it became less by March 30. The results in case 4740 were similar. Relatively slight agglutination occurred in the serum obtained on March 23, whereas in that obtained the next day, 24 hours after a series of 4 daily injections of the encephalitis serum were begun, there was marked increase in agglutinin content, only to recede by April 6, 12 days later.

The poliomyelitis strains were agglutinated almost as often and to the same degree as were the encephalitis strains, whereas the control strains were not agglutinated. The serum from normal persons had only slight agglutinating power over the different strains, or none.

The agglutination experiments show that while most of the encephalitis strains are immunologically alike, 51 of 65 being agglutinated specifically by the hyperimmune encephalitis and poliomyelitis serums, they vary considerably in their agglutinating properties, as indicated in the experiments with serums from patients, and that the encephalitis and poliomyelitis strains are similar immunologically.

SUMMARY AND CONCLUSIONS

Consistent results have been obtained in a series of undoubted cases of encephalitis, representing a wide range of types of the disease, and extending over a period of 4 years. Somewhat peculiar streptococci, much alike in the different cases, have been isolated constantly from infected tonsils, teeth, or nasopharynx during life, and from the brain after death. With this streptococcus in freshly isolated cultures, after as high as 44 rapidly made subcultures, and after a series of animal passages, characteristic symptoms and lesions of different forms of encephalitis have been reproduced in animals.

Peculiar neurotropic properties of the streptococcus have been noted in 4 species of animals, rabbit, monkey, mouse, and guinea-pig, following various methods of inoculation. Intracerebral inoculation, the method that has been so successfully used in virus studies in poliomyelitis, has given the best results. Results such as in these experiments were not obtained with green-producing streptococci from sources other than encephalitis. The organism has been demonstrated in the lesions which develop spontaneously in man, and which have been produced experimentally in animals, and proved absent in adjacent normal tissues and in the brains of persons and animals that died from other causes. It has been shown to possess specific antigenic properties. Most of the strains are immunologically alike, as determined by agglutination experiments with hyperimmune serums. The serum of patients with acute

forms of the disease agglutinated specifically the homologous and many heterogeneous strains. The requirements for proof of causal relationship between this streptococcus and encephalitis seem to have been fulfilled.

The organism varied greatly in size and shape, depending apparently on conditions of growth. Filtrates of nasopharyngeal washings, of emulsions of the brain in fatal cases, and of old anaerobic cultures have often yielded the streptococcus in culture and in positive animals. Large and extremely small forms, sometimes in the same chain, and large oval forms breaking into small forms have frequently been seen in cultures and have been demonstrated repeatedly in the lesions in undoubted cases of encephalitis (fig. 15).[1] Therefore, may it not be possible that the streptococcus with which I have been working is the larger aerobic, antigenic form of the smaller, strictly anaerobic, relatively nonantigenic form isolated by Loewe and Strauss,[5] Thalhimer,[6] and Maggiore and Sindoni,[7] and considered by them and others the causative agent in encephalitis?

[5] Loewe and Strauss: Jour. Infect. Dis., 1920, 27, p. 250.
[6] Arch. Neurol. & Psychiat., 1922, 8, p. 286.
[7] Pediatria, 1920, 28, p. 985.

HYDROGEN-ION STUDIES

XI. PREPARATION OF DRY ANTITOXIN AND AGGLUTININ POWDERS

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ANTITOXIN POWDER

Hirsch,[1] and Hirsch and LeCount,[2] have described the preparation of dry powders with specific immune properties (precipitin, amboceptor). Further work has demonstrated that a similar powder with antitoxin properties (diphtheria) can be prepared from immune horse serum. Soon after antitoxin became important in the treatment of diphtheria, dry antitoxin preparations (powders) were made by evaporating directly immune horse serum. Rosenau [3] was among the first to make such preparations. The hard flakes of dried serum were powdered in a mortar. The powder so obtained was kept dry with phosphorus pentoxide in suitable sealed containers, and stored in a cool, dark place. Hartley, Eagleton, and Okell [4] have made similar immune serum and complement serum preparations by evaporation. Their method in principle is essentially that described by Martin.[5] No attempts at purification are made with such preparations. The preparation of a dry powder with antitoxin properties, as described here, does represent a refining process, although no claims are made at present for the degree of purification. The details are as follows. Clear, potent serum [6] is obtained sterile from aseptically drawn and clotted horse blood. To a given volume N/100 HNO_3 is added (about 1 c c. per c c. of serum) until the P_H of the mixture is 6.8. A faint turbidity occurs followed by flocculation on standing in the icebox overnight. The liquid is filtered, and to the clear filtrate enough more N/100 HNO_3 is added (about 1.75 c c. per c c. of serum) until a maximum turbidity is produced. On standing in the icebox again a flocculent precipitate

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[1] Jour. Infect. Dis., 1923, 33, p. 470.

[2] Ibid., in print.

[3] Bull. Hyg. Lab. Wash., No. 21, 1905, p. 50.

[4] Jour. Path. & Bacteriol., 1923, 26, p. 53.

[5] Ibid., 1896, 3, p. 507.

[6] A supply of diphtheria immune horse-serum and standardized diphtheria toxin was obtained through the courtesy of Dr. E. M. Houghton of Parke, Davis & Co., Detroit.

forms and is removed by filtering. An equal volume of saturated ammonium sulphate is added to the clear filtrate, the precipitate is collected on soft filters, washed thoroughly with a half saturated ammonium sulphate solution, and then with saturated sodium chloride solution. The antitoxin substance dissolves in the sodium chloride solution and is again precipitated by half saturation with ammonium sulphate. The precipitate is collected on soft filters, drained thoroughly, and then dissolved in a volume of distilled water equal to about twice that of the original serum. To this clear and slightly opalescent liquid are added small volumes of saturated sodium chloride solution and N/10 HCl. A white flocculent or curdy precipitate forms. This is collected by centrifuging, and the supernatant liquid set aside for more to separate out, or more salt solution and N/10 HCl are added depending on precipitation tests when these reagents are added to a small amount of the liquid in a test tube. When the liquid volume is large the contained substances are concentrated by precipitation with ammonium sulphate, then dissolved in a small volume of distilled water and precipitated again by adding small amounts of saturated sodium chloride solution and N/10 HCl. The white precipitate, thoroughly centrifugated, is dried on watch crystals in a vacuum desiccator, most of the salt creeping out along the edges during the early stages of drying. Dried to a putty-like firmness, the material is divided into small particles, and when thoroughly desiccated is powdered in a mortar.

The light, white, starchy powder is soluble to a certain extent in water, forming a slightly opalescent liquid. The addition of a few drops of N/10 NaOH increases markedly its solution, and with good preparations a clear, limpid fluid is obtained. In testing the solution of these dried powders, a certain portion was found to be insoluble in distilled water or dilute alkali. This portion seems to separate out as the first precipitating particles, and so far as immune properties are concerned, it may be regarded as inert substance. Satisfactory preparations are made from such fractions by digesting with water sufficient to dissolve the soluble portion, and filtering. The dissolved substances are recovered from aqueous solution by precipitating with N/10 HCl and saturated sodium chloride solution. The precipitate is removed by centrifuging, and dried again in a vacuum desiccator.

Alkali added to increase the solubility of the powder brings the liquid to a certain reaction at which flocculation of the protein substance takes place. More alkali clears the liquid. Such a reaction is charac-

teristic of a colloid passing through a P_H range that includes its specific iso-electric point, and near this point exhibits the flocculation phenomenon. The substance with antitoxin properties then is precipitated and dried as a protein-acid salt. The solubility of the acid salt in water is considerably less than that of its alkaline salt, and probably the dissociation of the substance is quite similar.

The antitoxin strength of these powders was determined in guinea-pigs weighing approximately 250 gm. according to the method whereby the amount in solution necessary to neutralize an L + dose of diphtheria toxin is determined. For one powder the antitoxin unit so measured is 0.38 mg. dry weight; for another it is 0.35 mg.

Immune serums and serums with complement activity have been dried directly and preserved as such for some time. No attempt at purification is made with such preparations. The powders prepared according to the method described here are refined products although no claims at present are made for their degree of purity. The reduction to dry powder form of substances, with specific biologic properties may have considerable importance because of their stability, especially when solutions of such substances deteriorate. Such reference is made recently in a report by Best and Scott [7] on the preparation of insulin as a dry powder. It may be that dry powders with antitoxin properties can be preserved for considerable time without deterioration, and if this proves true, such preparations may have value commercially.

AGGLUTININ POWDERS

Certain modifications of the procedure originally given for preparing precipitin and hemolysin powders have improved the preparations and with the modified technic dry agglutinin powders readily soluble and actively potent in solution have been obtained quite regularly. Many experiments, notably those by Pick,[8] have demonstrated that the substances with immune properties are carried in the globulin fraction of serum; the agglutinins, quite regularly in the euglobulin, or the true globulin portion. Dry substances, which dissolved give specific immune properties to the solute, have been prepared by direct evaporation of immune serums. Recently Hartley, Eagleton and Okell,[9] made such preparations. No attempt at separating or refining the globulin fractions is made with these methods.

[7] Jour. Biol. Chem., 1923, 57, 722.
[8] Beitr. z. Chem. Physiol. u. Path., 1901, 1, p. 351.
[9] Jour. Path. & Bacteriol., 1923, 26, p. 53.

In my experiments, sterile citrated plasma of rabbits immune against typhoid, paratyphoid A, and paratyphoid B bacilli, respectively, has been used. The plasma is brought to a reaction of about P_H 6.8 with N/100 HNO_3. A flocculation or separation of a fibrin-like clot takes place on standing. The liquid is filtered, and to approximately each 5 c c. volume of the clear filtrate 2 c c. of saturated sodium chloride and 1.5 c c. N/10 HCl are added. A white flocculent precipitate forms at once, which is filtered off. The clear filtrate is half saturated with ammonium sulphate; the precipitate is collected on soft filters and washed thoroughly with a half saturated aqueous solution of ammonium sulphate and then with a saturated aqueous solution of sodium chloride. The precipitate on the filter paper is dissolved in a volume of water equal to about twice that of the original plasma. To approximately each 15 c c. of the clear or slightly opalescent liquid, about 0.25 to 0.5 c c. of N/10 HCl is added, and the white flocculent precipitate forming is filtered off. Solutions of this fraction do not agglutinate homologous bacteria. Saturated sodium chloride solution is then added slowly to the clear filtrate until a faint turbidity appears, and then from 0.25 to 0.5 c c. of N/10 HCl to approximately each 15 c c. of the filtrate. A white flocculent precipitate forms and settles out rapidly, more on standing. This is collected by centrifuging, dried thoroughly in a vacuum desiccator, and reduced to a light white powder, the aqueous solution of which agglutinates homologous bacteria in high dilutions.

A typhoid agglutinin dry powder was prepared in this way also with plasma obtained from a typhoid fever convalescent.

A test with typhoid agglutinin powder from rabbit plasma, stored in a sealed glass container in the icebox for 5 weeks, demonstrated that the original substance manifests its potency even in dilutions containing only 0.0035 mgm. per c c. The results of tests with paratyphoid A agglutinating powders were similar, a faint trace of agglutination being present in a dilution containing only 0.0022 mgm. per c c. The undiluted solution of the powder, specific for typhoid bacilli agglutinated these bacilli promptly and completely and to some extent also paratyphoid strains. The same is true as regards the effect of undiluted solutions of the paratyphoid powders toward typhoid bacilli. This is comparable to the well-known group agglutination of immune serums specifically potent in high dilutions and nonspecifically potent in low dilutions for closely related strains.

The results with a preparation stored in the laboratory for a period of 5 weeks suggest that the solids when properly prepared and stored are stable. Tests after longer periods will be made.

In the preparation of these dry powders with specific immune properties it seems that the solubility of the dried substances is determined, in a measure at least, by the proper ratio of salt and hydrogen-ion concentration in the final precipitating mixture. If the reaction be made too acid the dried substance is likely to become insoluble, and consequently inert. So far this ratio has been approximated only, but subsequent experiments are contemplated in the hope of establishing these relationships more precisely.

SUMMARY

A dry powder with antidiphtheriatoxic properties has been prepared, and dry powders of the antitoxins in all probability may be prepared from specific immune serums according to the method outlined.

Dry powders with active agglutinating properties have been prepared from rabbit and human plasma according to a technic modified somewhat from the method described for the preparation of precipitin and hemolysin powders.

The dry powder so prepared is a protein-acid salt readily soluble in water, and in solution actively potent.

Agglutination has been obtained with mixtures of homologous bacteria in dilutions containing as little as 0.0035 mgm. and 0.0022 mgm. of dry weight per c c.

One of these preparations has been stored about 5 weeks without loss of potency.

FOOD ACCESSORY FACTORS (VITAMINS) IN BACTERIAL GROWTH

VIII. RELATION OF SUBSTANCES FORMED BY B. COLI TO THE GROWTH OF YEAST

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It is generally conceded that the higher animals when placed on a diet lacking certain growth stimulating substances are unable to synthesize these substances themselves, and within a short period of time develop definite lesions which tend to disappear when these substances are added to their diet.

Bottomley [1] maintains that the higher plants as well are unable to form such substances, but obtain them from the soil, where they are generated by certain bacteria.

Jones [2] confirmed the work of Bottomley and showed that the addition of broth cultures of bacteria to peat, which was then used to fertilize poor soil, gave a marked increase in size and greenness to vegetables grown thereon as compared with controls grown on soil to which no organisms had been added.

Subsequent investigation has led to few definite conclusions so far as the bacterial origin of vitamins is concerned.

Lloyd [3] maintains that the meningococcus requires vitamins for its growth, while Shearer [4] reports the presence of an "accessory food factor" in the nasal secretions which greatly stimulates the growth of several bacteria, both pathogenic and nonpathogenic.

Davis [5] reports that the influenza bacillus requires in addition to all other food substances, a vitamin-like substance.

Mueller [6] maintains that several pathogenic organisms require growth stimulating substances.

Terrigo and Barnett [7] demonstrated a great acceleration of bacterial growth by adding water soluble B extract to old cultures of bacteria.

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[1] Proc. Roy. Soc., 1917, 389, p. 481.
[2] Abstr., Bacteriol., 1917, 1, p 43.
[3] Jour. Path. & Bacteriol., 1916, 21, p. 113.
[4] Lancet, 1917, 192, p. 59.
[5] Jour. Infect. Dis., 1917, 21, p. 392.
[6] Proc. Soc. Exper. & Biol. & Med., 1920-21, 18, p. 14, 225.
[7] Abstr., Bacteriol., 1921, 5, p. 5

Willaman[8] concludes that a small amount of plant decoction must be added to the synthetic medium prepared by him before Sclerotinia cinerea is capable of continued growth.

Wollman and Vagliano[9] confirmed earlier work showing that neither vitamin A nor B can be produced by B. bulgaricus or Amylomucor, while Leichentritt[10] concludes that the accessory food substances which are indispensable to man and animals are also necessary to bacteria.

On the other hand, Pacini[11] found that the typhoid bacillus elaborated a vitamin in cultures which will promote the growth of animals.

Eijkman[12] reports that B. coli cultivated in a medium containing an antineuritic factor remains devoid of this vitamin, as determined by feeding the washed organism to polyneuritic fowls.

In the course of experimental feeding Torrey and Hess[13] found no change in the putrefactive bacterial flora of scorbutic guinea-pigs at any time during the experiments. Creekmur,[14] on the other hand, reports a total reduction in the number of viable bacteria and a disappearance of streptococci in the fecal flora of rats on a vitamin A-free diet.

From the work cited it is evident that the soil bacteria afford a promising field of investigation. Because of its wide distribution in nature and the favorable results produced on plant growth by manuring the soil, B. coli-communis was chosen for the following experiments in an attempt to find an organism which can synthesize accessory growth substances on a vitamin-free medium.

The following medium was prepared, all substances being chemically pure: asparagin (Merc) 3.4 gm.; calcium chloride, 0.1 gm.; dextrose, 20.0 gm.; magnesium sulphate, 0.2 gm.; potassium phosphate (K_2HPO_4) 1.0 gm.; sodium chloride 5.0 gm.; sterile distilled water to 1 liter.

These substances were dissolved by boiling for 3 minutes, the original volume restored by addition of sterile distilled water and the reaction adjusted to P_H 7.4. The medium was then tubed in 5 c c. quantities in glass tubes. All glassware used had previously been thoroughly washed in $\frac{N}{1}$ NaOH, carefully rinsed in sterile distilled water and autoclaved at 20 pounds of steam pressure for 30 minutes. After tubing, the medium was again autoclaved at 20 pounds of pressure for 30 minutes.

Two strains of B. coli were used in these tests, one from the feces of a healthy adult, and the other a practically pure culture from the

[8] Jour. Am. Chem. Soc., 1920, 42, p. 549.
[9] Compt. rend. Soc. de biol., 1922, 86, p. 832.
[10] Berl. klin. Wchnschr., 1921, 58, p. 631.
[11] Jour. Biol. Chem., 1918, 34, p. 43.
[12] Ibid., 1922, 50, p. 311.
[13] Proc. Soc. Exper. Biol. and Med., 1918, 15, p. 74.
[14] Jour. Infect. Dis., 1922, 31, p. 461.

urine of a patient suffering with pyelitis. The results obtained were identical in both series of experiments.

Before inoculating the medium the bacteria were grown on nutrient broth for 3 generations with transplants every 24 hours. At the end of this time a tube of the synthetic medium was inoculated with 1 loop (about 1/30 c c.) of the broth culture. Thereafter transplants were made on the synthetic medium every 48 hours.

As controls, B. coli on nutrient broth and washed yeast cells on the synthetic medium and on dextrose broth were employed. As shown in a previous paper [15] this medium does not afford continuous growth for washed yeast cells when transplanted every 48 hours. The yeast was grown for 48 hours on dextrose agar slants, washed and thrown down in sterile salt solution in the centrifuge 3 times before using, and the inoculation completed within 1 hour after centrifugation. Aqueous

TABLE 1

RESULTS OF INOCULATION OF TUBES OF MEDIUM WITH B. COLI AND YEAST

Synthetic medium + B. coli	continued growth
Synethetic medium + yeast	no continued growth
Synthetic medium and carrot extract * / Synthetic medium and yeast extract * + B. coli	continued growth
Synthetic medium and yeast extract / Synthetic medium and carrot extract + Yeast	continued growth
Synthetic medium and B. coli (68th generation filtered) + yeast	continued growth

* Extracts in 1: 100—1: 500 solution.

extracts were prepared from grated carrot and Fleischman's yeast, filtered through a Berkefeld filter and added singly to the synthetic medium in concentrations of 1 : 500-1 : 100. The latter tubes were also used as controls when inoculated with yeast. Transplants were then made as infrequently as every 10 days in the case of yeast, and every 24 hours in the case of colon controls.

The 68th generation of B. coli was planted in a flask containing 1 liter of synthetic medium. At the end of 48 hours this medium was filtered through a Berkefeld filter using tap water pressure, the reaction adjusted to P_H 7.4, tubed in 5 c c. lots and autoclaved.

Yeast was planted on this medium, using the same technic as previously described. The results are given in table 1.

Growth was tested on dextrose-agar slants in the case of yeast and on plain agar slants in the case of B. coli, using one loop of the inoculated medium in all cases.

[15] Ibid., 1923, 32, p. 153.

Contamination of the tubes was frequently tested by the use of endo plates in the case of B. coli and dextrose-agar plates in the case of yeast. All tubes contained pure cultures of the organism in question throughout the experiment.

In all cases, the transplants were carried through at least 50 generation when continued growth is recorded, without diminution of luxuriance of growth after the first few generations except as reported.

It was noted that transplanting B. coli from one lot to another of the synthetic medium produced a temporary diminution in the luxuriance of growth, but this was overcome within a few generations. In this connection it might be added that although various lots of mediums were prepared as nearly alike as possible that a considerable variation in color was frequently observed after autoclaving. When autoclaving for a short time the medium was colorless, while continued autoclaving resulted in its turning a straw color or even brown, undoubtedly the result of a splitting of the dextrose in the presence of inorganic salts and alkali. For uniformity we autoclaved only as described, and in these cases the medium was practically colorless.

The growth of B. coli on the same lot of synthetic medium showed slight decrease in luxuriance with each transplant for the first 3-4 generations until macroscopically a 48-hour culture resembled in luxuriance a 24-hour culture of B. dysenteriae on broth. After this no further diminution was noted. No increased luxuriance of growth followed the addition of either of the water extracts.

On the other hand, in all cases in which continued growth is reported for yeast a luxuriant growth was obtained, so far as was determined grossly, equaling that of its growth on dextrose broth.

DISCUSSION

From these experiments it is evident that the growth of B. coli on synthetic medium for such length of time would result in the dilution of any substance carried over from the first generation to such an extent that it would be practically negligible (approximately 150^{-267}), and the presence of any additional substance or substances at the end of the experiment could be accounted for only as the metabolic products of the organism in question.

That such substance or substances were formed during the growth of B. coli is at once rendered apparent when the filtered medium of the 68th generation of this organism was inoculated with washed yeast cells. Continuous growth of yeast was then obtained as contrasted with

the short duration of growth obtained on the synthetic medium on which B. coli had not previously been grown.

Although from these experiments these newly formed substances are apparently closely related to the food accessory substances found in yeast and carrot extracts, whether they are identical with or closely allied to one of the known vitamins cannot be definitely stated as no feeding experiments on animals were made. These experiments are being carried out at present and should demonstrate to some extent the nature of this substance.

SUMMARY

The synthetic medium employed in the experiments here recorded permits the continued growth of B. coli-communis, but not that of yeast.

The addition of aqueous extracts of autolized yeast and grated carrot to the medium in high dilutions permits the continued growth of yeast but causes no increased growth of B. coli.

The filtrate of this synthetic medium, on which B. coli has been grown for 68 generations, permits the continued growth of yeast.

B. coli is apparently capable of continued growth on a vitamin-free medium, and by its growth thereon synthesizes a substance or substances which permit the continued growth of yeast on the filtrate.

EXPERIMENTAL HUMAN INOCULATIONS WITH FILTERED NASAL SECRETIONS FROM ACUTE CORYZA

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A review of the literature on this subject reveals the great divergence of results obtained by various investigators.

Kruse [1] succeeded in producing colds experimentally with a filtrate obtained from the nasal secretions of persons suffering with acute coryza.

Foster [2] succeeded in confirming the experiments of Kruse. Cultivating the filtrate by the method of Noguchi, he obtained a minute active organism visible with the dark field, which he states he successfully transplanted to the 4th generation, and which when then inoculated into the nostrils of 11 volunteers produced acute coryza in all.

Schmidt [3] from a series of 196 filtrate inoculations from 16 different subjects suffering with coryza concludes that his investigations do not support the filtrable virus theory of "colds."

Branham and Hall [4] state that in attempting to cultivate filtrable viruses from nasopharyngeal secretions in colds and influenza on the various mediums employed by them, no bodies were found in the cultures which could not be found also in those from normal persons, in controls, in all simple mediums examined, and on blank slides. This work does not support the work of Foster,[2] who contends that definite bodies were found when cultivated according to Noguchi's method.

Bloomfield [5] states that in a review of the literature he has found no convincing evidence that any known organism is the primary cause of colds, and cultural studies in his report fail to show in uncomplicated

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[1] Münch. med. Wchnschr., 1914, 61, p. 1547.
[2] Jour. Infect. Dis., 1917, 21, p. 451; Jour. Am. Med. Assn., 1916, 66, p. 1180.
[3] Deutsch. med. Wchnschr., 1920, 46, p. 1181.
[4] Jour. Infect. Dis., 1921, 28, p. 143.
[5] Bull. Johns Hopkins Hosp., 1921, 32, p. 121.

cases any variation in the flora which would enable one to select any organism or group of organisms as the cause of common colds.

Olitsky and Gates [6] have isolated an organism, Bacterium pneumosintes, from the nasopharynx of patients suffering from uncomplicated influenza. This when cultivated and injected into the trachea and nasopharynx of rabbits and guinea-pigs causes the clinical symptoms of influenza and seems to confer a certain degree of immunity to the rabbits, in some cases as long as 14 months. The organism is filtrable in the sense that it will pass through an ordinary porcelain filter, but it can be seen microscopically.

Lister [7] cultivated on Smith-Noguchi medium an organism corresponding to Bacterium pneumosintes and recovered it in nasopharyngeal washings from a volunteer who was taken sick with influenza following inoculation.

Olitsky and McCarty [8] conclude that the nasal secretions of a person suffering with acute coryza when taken from 3-18 hours after the onset contain a filter passing incitant and that common colds and epidemic influenza are separate and distinct diseases. They failed to find a specific exciting cause of coryza.

Of the factors believed to be the exciting cause of acute coryza by the various investigators, a filtrable virus appeared to be the most promising of positive results. It was our endeavor therefore to repeat as well as to extend experimental work in this field, with a view to acquiring more definite data, especially in connection with the nature of any virus which might be present.

In selecting cases for these experiments we limited ourselves to uncomplicated coryza which appeared during the fall, winter, and spring; and of these we endeavored to select the more virulent type, therefore apparently the most infectious.

The nasal secretions of persons suffering with such coryza were collected, from 6 to 144 hours after the onset, in a container which had been thoroughly sterilized and kept from contamination so far as possible; they were mixed with sterile salt solution in varying dilutions, from 1:10 to 1:50, and the mixture was shaken with glass beads in a small jar until homogeneous and free from lumps of mucus.

A record was made of clinical data with special reference to (a) history of recent exposure to acute coryza; (b) severe chilling as the

[6] Jour. Exper. Med., 1922, 35, p. 1.
[7] South African Medical Record, 1922, 22, p. 434.
[8] Jour. Exper. Med., 1923, 38, p. 427.

result of temperature changes or insufficient clothing; (c) evidence of contagion among associates. Smears of the secretions were then made and stained by Gram method and methylene blue.

Aerobic blood-agar plates were inoculated with a loop of this material, examined at the end of 24 and 48 hours, and the organisms identified.

The solution was then passed through a Berkefeld filter which was impermeable to ordinary cocci using tap water pressure—a procedure never taking more than one hour—and at the completion of which, smears and aerobic and anaerobic blood-agar cultures were made of the filtrate. In every case these cultures from the filtrate—both aerobic and anaerobic—remained sterile for 96 hours. On all of the smears made from the filtrate were found coccoid bodies, apparently those which were believed by Foster [2] to be the specific exciting cause of acute coryza.

These coccoid bodies were quite small, though visible under oil immersion, round or slightly flattened, quite distinct in outline, and possessed a marked tendency to occur in pairs, although they also appeared singly or in clusters. They did not appear to possess true motility and retained the Gram stain. They could be demonstrated in filtrates from the nasal washings of normal persons and on stained clean glass slides as readily as in the filtrates from the nostrils of persons suffering with acute coryza. Beyond the mediums mentioned no attempt was made by us to cultivate these bodies, for we found no reason to believe that they played a part in the etiology of acute coryzas, or indeed were organisms at all.

Inoculations with the filtrate were made into the nostrils of volunteers free from coryza or catarrhal affections as soon after filtration as possible. In no case did this time exceed 24 hours after the collection of the secretions, which in the meantime were kept in the icebox. For inoculation purposes, we found it convenient to use sterile, drawn glass tubes plugged at one end with cotton, in which the filtrate was aspirated and the drawn end sealed in the flame. At the time of inoculation this sealed end was broken off, sterilized by the flame, and the liquid sprayed over the nasal mucosa of the volunteer. About 1 c c. of the filtrate was placed in each nostril by this method.

All infections of the respiratory tract following within 12 days of the inoculation were regarded as positive even though they did not later develop into a coryza. The inoculated person reported the infection as

soon as possible, and the clinical signs, as well as the history of exposure and contagion among associates, were recorded. When no symptoms had appeared by the end of the 12th day, the inoculation was recorded as negative.

The volunteers inoculated during these experiments were faculty members, students, or employees of the University of Illinois, College of Medicine or College of Dentistry, whose daily work brought them in contact with many persons from whom they, unknown to themselves, might have contracted coryza, or transmitted it to them.

In summarizing the observations, it is found that the onset dated back to exposure due to chilling of the body in 2 cases (18.18%), and to other coryzas in 1 case (9.09%). There was a negative history of exposure in 8 cases (72.72%). There was evidence of contagion among associates in 3 cases (27.27%).

Bacteriologically, the incidence of the various organisms which occurred in the unfiltered secretions is:

Staphylococcus albus in 9 cases (81.81%)
Micrococcus catarrhalis in 4 cases (36.36%)
Streptococcus nonhemolyticus in 4 cases (36.36%)
Streptococcus viridans in 3 cases (27.27%)
Bacillus influenzae in 1 case (9.09%)
Diphtheroids in 1 case (9.09%)
Pneumococcus (IV) in 1 case (9.09%)
Streptococcus hemolyticus in 1 case (9.09%)

The incidence of 2 organisms in the secretions is:

Staphylococcus albus and micrococcus catarrhalis in 2 cases (18.18%)
Staphylococcus albus and Streptococcus viridans in 2 cases (18.18%)
Staphylococcus albus and Streptococcus hemolyticus in 1 case (9.09%)
Staphylococcus albus and Streptococcus nonhemolyticus in 1 case (9.09%)
Streptococcus viridans and Streptococcus nonhemolyticus in 1 case (9.09%)

The incidence of three organisms in the secretions is:

Staphylococcus albus, Micrococcus catarrhalis, and diphtheroids, in 1 case (9.09%)
Staphylococcus albus, Streptococcus nonhemolyticus and B. influenzae in 1 case (9.09%)
Staphylococcus albus, Micrococcus catarrhalis, and pneumococcus (IV) in 1 case (9.09%)

The results of the 100 human inoculations are positive for bronchitis, 1 case (1%); coryza, 1 case (1%); influenza, 1 case (1%); and laryngitis, 2 cases (2%). There were 95 negative cases, or 95%, free from any respiratory infection following the inoculation.

TABLE 1

RESULTS OF EXPERIMENTAL INOCULATIONS

Subject					Secretions						Inoculations of Volunteers					
														Positives		
No.	Onset	Predisposing Factors	Course	Evidence of Contagion	Collected	Bacteriology	Dilution	No. of Volunteers	Date	No. of Negatives	Number	Onset	Predisposing Causes	Course	Evidence of Contagion	Bacteriology
I	Nov. 2, 1921	None	Moderate	None	Nov. 3, 1921 (20 hours from onset)	Staphylococcus albus, nonhemolytic streptococcus	25×	6	Nov. 4, 1921	6	0					
II	Dec. 12, 1921	Chilled while traveling on railroad train, night of Dec. 11	Moderate	All of roommates (3 in number) developed coryza within 7 days	Dec. 14, 1921 (36 hours from onset)	Staphylococcus albus, Micrococcus catarrhalis, Pneumococcus (IV)	25×	6	Dec. 14, 1921	5	1	Dec. 17, 1921	None	Mild laryngitis	None	Streptococcus viridans, Streptococcus nonhemolyticus
III	Dec. 13, 1921	Evening of Dec. 12 studied with friend suffering with acute coryza	Severe	None	Dec. 14, 1921 (12 hours from onset)	Staphylococcus albus, Streptococcus viridans	10×	6	Dec. 15, 1921	5	1	Dec. 15, 1921 1½ hours after inoculation	None	Mild influenza	None	Streptococcus viridans. Streptococcus nonhemolyticus, Dec. 18, 1921
IV	Jan. 3, 1922	None	Moderate	None	Jan. 3, 1922 (6 hours from onset)	Streptococcus viridans, Streptococcus nonhemolyticus	25×	7	Jan. 4, 1922	6	1	Jan. 7, 1922	Fatigue from loss of sleep	Mild laryngitis	None	Staphylococcus albus, Streptococcus viridans
V	Feb. 4, 1922	None	Severe	Wife and 2 children developed similar coryza within 7 days	Feb. 10, 1922 (144 hours from onset)	Staphylococcus albus, Streptococcus nonhemolyticus, B. influenzae	25×	7	Feb. 11, 1922	7	0					
VI	Feb. 12, 1922	Chilling because of insufficient bed clothing night of Feb. 11	Severe	None	Feb. 13, 1922 (24 hours from onset)	Streptococcus nonhemolyticus	30×	4	Feb. 14, 1922	4	0					
VII	Mar. 21, 1922	None	Severe	None	Mar. 22, 1922 (20 hours after onset)	Staphylococcus albus, Streptococcus hemolyticus	50×	10	Mar. 23, 1922	8	2	No. 5 on March 24 No. 8 on March 28	None None	Mild coryza Mild bronchitis	None None	Not taken Staphylococcus albus, Streptococcus viridans
VIII	Apr. 3, 1922	None	Severe	None	April 4, 1922 (12 hours from onset)	Staphylococcus albus, Micrococcus catarrhalis	25×	20	April 5, 1922	20	0					
IX	Apr. 17, 1922	None	Severe	None	April 18, 1922 (18 hours from onset)	Staphylococcus albus, Micrococcus catarrhalis	40×	14	April 18, 1922	14	0					
X	Oct. 17, 1922	None	Severe	Two sons developed coryza of similar type with in 5 days from onset	Oct. 20, 1922 (60 hours from onset)	Staphylococcus albus, Micrococcus catarrhalis, Diphtheroids	25×	10	Oct. 20, 1922	10	0					
XI	Dec. 14, 1922	None	Severe	None	Dec. 15, 1922 (12 hours from onset)	Staphylococcus albus Streptococcus viridans	30×	10	Dec. 15, 1922	10	0					

DISCUSSION

Because of the small number of positive results following the inoculations, we found it unnecessary to employ controls, and the variation in the 5% of cases recorded as positive convinces us that these were the result of causes entirely independent of the inoculations. In any group of persons selected at random during a time when an endemic of colds is present, a certain small percentage will develop some upper respiratory infection within a period of 12 days. This we believe fully explains the cases which we recorded as positive.

Though extensive experiments have been made on the bacteriology of acute coryza, the results obtained have been far from satisfactory. As our experiments were planned primarily to establish the status of a filtrable virus in their etiology, no human inoculations were attempted with any of the cultivated organisms.

In all cases examined a marked reduction of the bacterial flora occurred in the secretions with the predominance of one organism during the onset and early stages of the attack. When the secretion became purulent, usually from 36 to 48 hours from the onset, bacterial examination revealed a marked increase of the flora over the normal flora of health still with the predominance of one organism, usually Staphylococcus albus. When the secretions began to diminish, the bacterial flora diminished and gradually returned to that of health.

We believe this to be a point of some significance when considered with the negative results obtained on filtrate inoculation. Presumably it is an indication that the exciting factor is a specific, nonfiltrable organism which in some manner decreases the nasal flora during the onset and early stages of the attack, while its elimination during the course of the attack, together with its depressant action on the mucosa, permits a marked increase over the normal flora (still, however, with one predominating organism) and during convalescence a gradual return to the flora of health.

Although from bacteriologic examinations no satisfactory proof has as yet been produced which shows that coryzas are infectious, all clinical and epidemiologic manifestations point in this direction. One has but to note the occurrence of endemics and epidemics of acute coryza in civilized communities—the absence of which has often been noted in the arctic regions by explorers—to be convinced that the specificity of these infections is as clearly apparent as in many of the acute infectious diseases of established etiology.

Predisposing causes, such as seasonal changes, lowered resistance, exposure to cold and moisture, and individual anatomic variations have been pointed out by many investigators and must be kept in mind at all times, as should also the clinical variations observed from the type with but slight serious discharge and no systemic effects to the virulent type closely resembling influenza.

That coryzas frequently play a large part as predisposing causes of other acute or chronic infectious respiratory diseases cannot be questioned. This inter-relationship is frequently seen in influenza, pneumonia, and tuberculosis, in which the onset is said to date back to "a cold," and for this reason their prevention and treatment should play an increasingly large part in modern preventive medicine.

SUMMARY

In this series of experiments, nasal secretions were secured from 11 persons suffering with acute uncomplicated coryza. After being diluted and passed through a Berkefeld filter, these secretions were sprayed onto the nasal mucosa of 100 volunteers.

The experiments presented no convincing evidence indicative of a filter passing organism as the exciting factor in acute coryza. We believe the cases recorded as positive to be the result of factors independent of the inoculations.

During an attack of coryza definite variations were noted in the bacterial flora of the secretions. During the onset and early stages of the attack, there was a marked diminution of the total bacterial flora, with an equally marked predominance of one of the normal habitants—usually Staphylococcus albus.

During the purulent stage of the attack, a marked increase of all organisms over the normal flora of health was observed, although the predominance of one organism still remained. The later stages of the attack were marked by a gradual return to the normal flora of health.

OXYDASE ACTIVITY AND ISOLATION OF PURE CULTURES OF BACTERIA *

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Bacteria vary in their ability to oxidize p-amino-leuco-malachite green.[1] The conditions under which this chemical reaction is brought about by certain species of micro-organisms are quite definite and consist mainly of a proper substrate and a suitable supply of oxygen. That is, given a certain organism in a suitable medium containing the leukobase, under aerobic conditions in an incubator, the leukobase will be oxidized into its corresponding dye form. If the leukobase medium is incubated without inoculation of a microbe, oxidation does not take place. Thus, the definition of an oxydase has been satisfied, a resultant oxidation caused by an agent in the presence of molecular oxygen, the absence of which prevents this form of oxidation process. A substrate suitable for the purpose of studying the oxydase activity of bacteria, reported elsewhere, is fresh serum or blood, while meat infusion or peptone is not only lacking in fitness, but also neutralizes the favorable quality of serum in a quantitative fashion.

It was shown in my previous study that pneumococci and streptococci are capable of causing this form of oxidation, while none of 23 other bacterial species responded to the test. Since that time, p-amino-leuko-malachite green has been in constant use as an indicator in this laboratory, and the former results have been confirmed so far as repetitions have been made. It is established that the small gram-positive cocci, pneumococci and streptococci, under prescribed conditions, stand out as the highest oxydase producers; while the large gram-positive and all gram-negative cocci, as well as the gram-negative bacilli and most of the gram-positive bacilli, are either entirely lacking in the ability to oxidize this indicator or can do so only feebly. I do not wish to convey the impression that bacteria other than pneumococci and streptococci may or may not have oxidative activity as evidenced by some other indicator, but that the leukobase of p-amino malachite green lends itself not only to demonstrate oxydase activity of certain microbic

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[1] J. Exper. Med., 1923, 38, p. 291.

species, but also to serve as an aid for the isolation of pure cultures. The specificity of the reaction, though not ideal, is quite definite, as is shown in the previous work on liquid medium. However, to utilize oxydase activity of bacteria for the purpose of isolation of pure cultures, a solid medium is essential, and thus it became necessary to study the effect of oxydase activity on this form of medium. The 4 experiments given are designed to show the conditions necessary to demonstrate optimum oxydase activity on medium suitable for growth of single colonies.

Exper. 1.—*Effect of Different Ingredients in Agar Base.*—Plain broth and its constituents, meat infusion and peptone solution were

TABLE 1

The Effect of Agar Base on Oxydase Production

Medium P_H 7.4	Micro-organisms					
	P I	P II	P III	Streptococcus Hemolyticus	Streptococcus	Staphylococcus
Agar + sodium chlorid... 10% horse blood	+	+	+	±	±	0
Agar + 2% peptone..... 10% horse blood	++	++	+	+	+	0
Meat infusion agar..... 10% horse blood	++++	++++	+++	+++	++	0

In this table, as in those following, P indicates pneumococci; I, II, III, IV, the type. The number of plus signs has the usual significance, ++++ denoting the most intense reaction.

shown to inhibit or neutralize the oxydase production of pneumococci. For this reason exper. 1 was planned to determine their influence in an agar base. Three combinations were tested: (1) agar alone, (2) agar and 1% peptone and (3) the usual meat infusion agar; to each of these, the indicator in 1 : 5,000 dilution and 10% horse blood were added. Plates were poured and inoculated with the organisms, as shown in table 1. Much to my surprise, the meat infusion agar gave the best results and that of the plain agar the poorest; while the peptone supplied conditions of suitability between the two. These observations seemingly reveal a difference between the results reported on the effect of plain broth on oxydase production and those obtained here on solid mediums. There is some difference. In explaining the variation these facts must be kept in mind: (1) that serum neutralizes the effect of plain broth antagonistic to oxydase reduction quantitatively and (2)

that the greater the surface of exposure to molecular oxygen, the more rapid is the oxydase reaction. The latter fact was established by testing the rate of oxidation in the same volume of medium in different sized test tubes and flasks. The vessel having the greatest surface exposure always gave the most rapid oxidation. Therefore, although the concentration of blood in the medium is not sufficient to destroy or neutralize entirely the action of plain broth antagonistic to this reaction, I think this factor in combination with the maximum surface exposure to oxygen adequately explains the difference between the work previously reported, when the mediums used were in liquid state, and the observation made here on solid medium. A comparison of the abundance of

TABLE 2

EFFECT OF DIFFERENT CONSTITUENTS OF BLOOD

Medium P_H 7.4	Micro-organisms					
	P I	P II	P III	Streptococcus Hemolyticus	Streptococcus	Staphylococcus
Meat infusion agar + 10% whole blood	++++	++++	+++	+++	+	0
Meat infusion agar + 10% horse serum	0	0	0	0	0	0
Meat infusion agar + 10% washed cells	±	±	±	±	0	0
Meat infusion agar + hemoglobin from 10% washed cells	±	±	±	±	0	0

growth on these 3 mediums, as judged both by the size of the colonies and their number when inoculations were made from the same dilution of organisms, shows quite clearly that the oxydase reaction parallels the degree of rapidity of growth; for the best growth as well as the most intense color change was observed to be on the meat infusion blood agar.

Exper. 2.—*The Effect of Different Constituents of Blood.*—To detect the influence of different constituents of blood as a substrate on this oxidation reaction, the following were tested: whole defibrinated blood serum, red cells, and hemoglobin, each used in 10% concentration. The red cells, washed 3 times, were diluted to the original volume with normal salt solution, and 10% of this added to the agar base. Likewise, laked red cells as a source of hemoglobin were adjusted so that the same concentration of hemoglobin as was contained in the

10% cells was employed in the plates. The results (table 2) indicate that defibrinated blood supplies the necessary substrate for the reaction, while red blood cells and hemoglobin produce conditions slightly favorable, but serum is without visible stimulatory effect. Again, as before, the amount of growth in these different mediums may be at least partially responsible for the variation in oxydase production. For the best growth, as judged by the size of the colonies, was found on the whole blood plates.

Exper. 3.—*Effect of H-Ion Concentration.*—As it was impracticable to determine the H-ion concentration in 10% blood agar, colorimetric

TABLE 3

Effect of H-Ion Concentration

Meat infusion agar + 10% whole citrated horse blood

H-Ion Concentration		Micro-organisms					
		P 1	P II	P III	P IV	Streptococcus	Staphylococcus aureus
P_H 6.5	Growth	++	++	++	++	++	++
	Col.	++	++	++	++	+	0
P_H 7.0	Growth	+++	+++	+++	+++	+++	++++
	Col.	++++	++++	++++	+++	+++	0
P_H 7.5	Growth	innumerable	innumerable	innumerable	innumerable	innumerable	innumerable
	Col.	++++	++++	++++	+++	+++	0
P_H 8.0	Growth	+++	+++	+++	+++	+++	++++
	Col.	+	+	++	+	0	0
P_H 8.5	Growth	+	0	+	+	0	++
	Col.	0	0	0	0	0	0

Col. indicates color reaction.

estimation was first made on 10% serum agar, and the necessary amount of acid or alkali was added to a measured quantity of agar which had been found to produce a given H-ion concentration of the serum agar; and to this, then, 10% blood was added. The resultant H-ion concentration is of necessity an approximation, but of sufficient accuracy for the present purpose. Growth occurred in mediums of H-ion concentration from $P_H6.5$ to P_H8, the optimum being $P_H7.5$. The oxidation of the indicator does not quite parallel the degree of abundance of growth. The color intensity in both mediums of P_H7 and $P_H7.5$ was found to be about the same, while the growth was better in the latter. Again, in mediums of P_H8 and P_H7, the same degree of growth occurred, but the intensity of the color of the indicator was noticeably less in the alkaline medium. These results are the same as

those obtained in liquid mediums, in which it was found that the more acid the medium, within limits, the more intense was the oxidation of the indicator.

It will be noted in table 3 that the strain of streptococcus growing in medium of P_H8, although growing well, failed to oxidize the indicator. A similar reaction has been observed with several strains of streptococci, so that attempts have been made to use the variation of oxydase production at different H-ion concentrations as a means of differentiation between pneumococci and streptococci. A certain number of strains of streptococci have failed to oxidize the indicator in mediums of $P_H7.8$ or above, but although the strains of pneumococci used in the same medium always gave a positive reaction, it is not

TABLE 4

EFFECT OF DEXTROSE

Five % horse blood in meat infusion agar PH 7.4

Dextrose	Micro-organisms					
	P I	P II	P III	P IV	Strepto-coccus	Staphylo-coccus
0.2%	+++	+++	++	+++	+	0
0.5%	++++	++++	+++	++++	++	0
1.0%	++++	++++	+++	++++	++	0
2.0%	+++	+++	++	+++	+	0
0	+++	+++	++	+++	+	0

improbable that some strains of pneumococci may be found which, under the same conditions, would also give a negative reaction. Although differences are manifest, not at any P_H of the medium so far used has there been a clear line of demarcation. The intensity of the reaction can be modified, but the changes so produced are common to both species of cocci. It is possible that a different indicator or quantitative technic may reveal a constant difference between these two species.

Exper. 4.—*The Effect of Dextrose.*—Dextrose in liquid medium has been found to stimulate oxydase production of pneumococci. In order to estimate its effect on blood agar, varying percentages of dextrose were added to a base medium which did not in itself supply optimum conditions, namely, a 5% blood agar instead of the 10% usually employed. The results of these experiments (table 4) show that dextrose in concentrations of 0.5 or 1% definitely stimulate this reaction, while a greater or lesser percentage seemingly is without effect. Dextrose blood agar has therefore been used for routine work in the

laboratory for some time to advantage. It has, however, a slight disadvantage; growth is not supported as well when the medium is from 24 to 48 hours old as when freshly made. The indicator seems to be as sensitive, but the colonies are smaller, and consequently the color reaction is not as intense.

PRACTICAL USE OF THE INDICATOR MEDIUM

The indicator medium has been in use in this laboratory for over a year as an aid in the isolation of pneumococci, and although the differentiation is not ideal, its use has led to greater ease in picking colonies than has any other technic. The pneumococcus colony besides having its well known morphologic characteristics is dyed a faint blue and the medium surrounding it a deep purplish blue. In our experience, only two bacterial species consistently cause the oxidation of the indicator on this medium—pneumococci and streptococci. Our interest lay in a study of these two species, and in picking over hundreds of plates nothing but these small gram-positive cocci were found to produce a positive reaction. Other bacteria may be able to oxidize this indicator under the conditions here reported, but so far none has been observed.

The composition of the medium used as a routine is a 0.5% dextrose, 10% horse blood agar to which is added the indicator (p-amino-leuko-malachite green) to make a 1 : 5,000 dilution of the whole. The meat infusion agar base is made as usual; 1 pound of lean beef to a liter, 1% peptone, 0.5% sodium chloride and adjusted to P_H7.6. Sterilization of this medium may be done either in the autoclave under 15 pounds' pressure or on 3 successive days in free steam without any appreciable effect on its practicability. The leukobase is insoluble in water but soluble in acid. A solution, accordingly, was made by dissolving it in dilute hydrochloric acid and diluting to the desired volume by water. The indicator was stocked in a 1% solution after sterilization in an Arnold sterilizer. Indicator medium is made up when desired by adding the leukobase (2 cc. to 100 cc. of agar) and dextrose to make 0.5% to melted agar, and the mixture is then adjusted to the desired P_H. When cooled to 50C., 10% blood is added, and the plates are poured in the usual manner. Fresh plates give the best results.

DISCUSSION

Although this study deals with the practical application of an activity of certain species of organisms as a means of isolation of pure cultures,

it may be included in one of a series of investigations in which the action of bacteria on animal tissue is studied in the hope of unraveling some of the complexities in the relationship of action and reaction between host and bacteria. For example, as far as has been determined, this oxydase reaction depends on animal tissue as a suitable substrate. The question then naturally arises, especially when the marked species specificity and the fact that it is an oxidative process are considered, Is there a connection between oxydase production and pathogenicity and virulence? Certainly the infectious process of bacteria is essentially chemical in nature. The problem is to determine what chemical processes or products are significant.

It is to be emphasized that the purpose of this paper is a presentation of a method of determining oxydase action of bacteria as well as the practical use of this activity as an aid to diagnosis. The organisms studied were chosen at random and verified as to species only. In the foregoing tables, a certain variation can be noted among the organisms giving a positive reaction. The variation is commonly seen in routine picking, but so far has been of no value in differential diagnosis. The fact that such differences exist may be a clue to the possible differentiation by indicators more sensitive or selective than the one here described. On the other hand, the action of the staphylococcus as shown above is typical for this species: for at no time has a strain of this organism oxidized this indicator on a blood agar plate.

In our study on oxydases, it was noted that the serum from different animal species varied in suitability as a substrate for this oxydase reaction. For solid medium this question was not thoroughly studied. Horse blood was used because of availability and consistency of results. However, specimens of blood of rabbits and guinea-pigs were tested and found to give more pronounced reaction than horse blood: so that a 5% blood medium from the blood of these animals is about as efficacious as 10% horse blood.

SUMMARY

A new method for the study of oxydase production of bacteria has been given, and its use as a practical method for aid in isolation of cultures is described. The method depends on the oxidation of p-amino-leuko malachite green in a proper substrate in the presence of oxygen. The common factors which influence the reaction are, the necessity of whole blood in a meat infusion base, optimum H-ion concentration between P_H7 and $P_H7.6$, and dextrose in percentage of 0.5 or 1.

A NEW INDICATOR FOR TESTING REDUCING POWER OF BACTERIA *

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The work reported in this paper concerns the following two important uses of dyes in bacteriology: (1) to aid in the isolation of pure cultures, (2) to determine the oxidizing and reducing power of bacteria. Dyes are useful in the isolation of pure cultures through their ability to restrain the growth of certain bacteria and favor the growth of others. Thus, Endo made use of the differential property of fuchsin S; Conradi and also Krumwiede used brilliant green; Werbiski, China green; and Loeffler, malachite green. The oxidative and reducing power of micro-organisms may be determined by the use of certain dyes as an indicator of this activity. As a rule, the leukobases are used to indicate oxidation and the colored compounds to determine reduction

Oxidative action of bacteria has received little attention. I [1] recently reported the results of studies on oxydase production of a number of pathogenic micro-organisms. It must be clearly borne in mind that oxydases and their mode of action are not well understood. It is known that oxydases are capable of oxidizing indirectly by changing molecular into active oxygen, in which state it combines freely. Karczag and Hajos,[2] however, have shown that B. tuberculosis, B. anthracis and B. subtilis oxidize the carbinol of fuchsin S into the dye. On the other hand, it is well known that bacteria are able to cause a reduction, as judged by changing a dye into a carbinol or leukobase. The compounds generally used for this purpose are methylene blue, neutral red, litmus and the sulphindigotate (Weyl).

In the course of another study, I observed that a green dye (p-nitro malachite green) is changed to a red one in the presence of growing B. typhosus. It is known that the nitro group in acid solution is reduced to an amin, and it is therefore assumed that the change of color from

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[1] J. Exper. Med., 1923, 38, p. 291; J. Infect. Dis., this issue, p. 407.

[2] Bioch. Ztschr., 1923, 138, p. 408.

green to red indicates that the para-nitro malachite green was reduced to the para-amino malachite green by the action of B. typhosus.

Malachite green itself and other members of this group which varied by possessing different radicals in the para position analogous to the p-nitro compound were tested. No color alteration, however, was noted with p-chlor, p-hydroxy, p-methyl, p-ethyoxy and p-methyoxy malachite green, other than the formation of the carbinol compound, a change from a colored solution to a colorless one. How much this change was due to the action of B. typhosus, to the plain broth or to the serum was not determined. It was noted that all compounds alike became colorless in fresh horse serum. A transition was made from dye to carbinol by the action of serum; a reaction the same as that reported by Karczag. These malachite green derivatives were not changed in color by the reducing action of B. typhosus. The compounds may have been reduced, but there occurred no visible evidence. It is the nitro group in the p-nitro malachite green which lends itself as an indicator for the detection of reducing power, for the reason that the product of reduction is a compound of a different color than the original.

TESTS WITH VARIOUS BACTERIAL SPECIES

The action of B. typhosus on this compound naturally led to the testing of other bacterial species to determine whether they were able to reduce malachite green from its nitro to an amin compound. The organisms tested were grown in two kinds of medium, the one plain broth made from lean beef, 1 pound to a liter, and 1% Difco peptone, adjusted to P_H7.4, and the other 10% horse serum in normal salt solution. Normal horse serum was included because I have observed that it makes a good medium in which to test the oxydase production of bacteria. The indicator, p-nitro malachite green, was added to the medium in a dilution of 1 : 10,000. Fourteen different bacterial species, consisting of 48 strains in all, were studied. Most of the strains were stock cultures obtained from different laboratories. Each culture was tested so as to verify its classification. The results of the study of these various species are shown in table I. On plain broth, all members of the colon-typhoid group tested, except B. alkaligenes, reduced the nitro compound. On medium containing serum, however, B. typhosus was the only species of this group which gave a strongly positive reaction. The fact that B. alkaligenes is unable to reduce this

indicator may be explained, perhaps, by the fact that nitro compounds are reduced differently in alkaline than in acid solutions.

Of the five species of cocci examined, meningococci possess the greatest reducing power, as judged by this indicator; Streptococcus hemolyticus and Staphylococcus aureus and albus exhibit similar reduction, but to a less degree. The specificity of the action is clearly indicated by the fact that none of the 11 strains of pneumococci or the 4 strains of B. diphtheriae give any reduction either in plain broth or in horse serum.

OXYDASE ACTIVITY AND REDUCING POWER

An interesting and perhaps significant analogy is afforded between the oxidative and reducing activity of bacteria. In the light of my other work,[1] it was shown that certain species have greater oxydase production activity than others. Thus, it was observed that pneumococci are more active than Streptococcus viridans or Streptococcus hemolyticus and other streptococci studied. It was also shown that certain other bacteria, as judged by the indicator p-amino malachite green, were incapable of manufacturing oxydases. A correlation made between the action of micro-organisms on p-amino-leuko malachite green and p-nitro malachite green suggests new developments in our knowledge of the activities of bacteria. For it was found that those which reduced the nitro compound lacked the power to cause oxidation of the leukobase under practically the same conditions. Therefore, bacteria arrange themselves into 3 different groups: (1) those with great reducing power, (2) those of great oxidizing power, and (3) an intermediate group which exhibits both oxidative and reduction potentials. This relationship is suggested only by the differential action of bacteria on these two indicators. It is a question, however, whether an organism which possesses the faculty of strong oxydase production may demonstrate in a strict sense a high oxidation-reduction potential.

DISCUSSION

The oxidizing and reducing power of bacteria as shown by their influence on dyes has recently been reported by Karczag[3] and his collaborators. This work was published under the title "Electropie" and has to deal with the action of various groups of chemicals, organic and inorganic, and also of bacteria on dyes including the color base (carbinol) and the leukobase. The process of change of these indicators

[3] Bioch. Ztschr., 1923, 138, p. 345.

from one form to another was called by these investigators "chemoskopie." Although they speak of electrical potential and alteration of this force by different substances, they do not correlate the reaction with the oxidation reduction potential. Karczag's phenomenon of "chemoskopie" is an example of oxidation reduction potential, as the transformation of dyes affords a good indication of this potential. Hence, there is little need of this new term. This investigation, however, is a step toward a better knowledge of a biologic reaction of fundamental importance.

TABLE 1

ACTION OF CERTAIN MICRO-ORGANISMS ON PARA-NITRO MALACHITE GREEN

Species of Micro-organisms	No. of Strains	Plain Broth P_H 7.4			10% Horse Serum		
		Intervals in Hours			Intervals in Hours		
		24	48	72	24	48	72
B. lactis aerogenes	1	2	4	4	0	0	0
B. friedländer	1	2	4	4	0	0	0
B. coli communior	2	2	4	4	Tr	Tr	Tr
B. coli communis	2	4	4	4	Tr	Tr	Tr
B. alkaligenes	1	0	0	0	0	0	0
B. proteus vulgaris	1	3	4	4	0	0	0
B. pyocyaneus	1	Tr	4	2	0	0	0
B. dysenteriae Shiga	3	Tr	2	2	0	0	0
B. dysenteriat Strong	1	Tr	4	4	0	0	0
B. dysenteriae Hiss	2	2	4	4	0	0	0
B. paratyphosus A	1	2	4	4	0	0	0
B. paratyphosus B	1	2	4	4	0	0	0
B. typhosus	3	2	4	4	2	2	3
Meningococcus	9	1–3	2–4	2–4	1–2	2–4	2–4
Streptococcus hemolyticus	2	0	2	2	1	1	1
Staphylococcus albus	2	Tr	Tr	Tr	Tr	Tr	Tr
Staphylococcus aureus	2	Tr	1	1	Tr	Tr	Tr
Pneumococcus	11	0	0	0	0	0	0
B. diphtheriae	4	0	0	0	0	0	0
Control	..	0	0	0	0	0	0

Tr indicates trace; 1, 2, 3, 4, respectively, 25, 50, 75 and 100% intensity of red color; 0, no red color visible. In broth the green color of the dye remained constant if not changed to red. Concentration of the dye 1:10,000.

Observations made by Karczag and Hajos [2] that some bacteria change fuchsin S into the carbinol form, whereas other bacteria lack this action but transform the carbinol into the dye, are similar to the results reported in this paper with p-nitro malachite green. The action of both indicators makes it clear that certain bacterial species are more capable of causing reduction than others. The reaction that takes place, however, varies according to the dye used as an indicator. For example, fuchsin S is reduced to the carbinol form, while p-nitro malachite green is first reduced to the nitro carbinol, then to the carbinol of the amino compound, and this is finally oxidized to the dye. The

former is a tautomeric change, while the latter is a structural or chemical transformation with an associated color alteration.

The ability of bacteria to reduce the nitro compound, described in this brief paper, finds also an analogy in the work of Lipschitz [4] who studied the mechanism of oxidation in animal cells. This investigator used m-dinitrobenzene to measure the reducing power of frog muscle, and found that the muscle cell transformed the colorless m-dinitrobenzene to the yellow m-nitrophenylhydroxylamin. This is clearly a similar reduction to that of p-nitro malachite green by B. typhosus and other bacterial species. In the case of the frog muscle, the reaction is quantitative; that is, the amount of colorless compound reduced can be estimated from the intensity of the yellow amin compound formed. The similarity between the action of bacterial cells and animal cells on nitro compounds suggests a step toward a better understanding of the action of bacteria in vivo. This may be true whether or not the action is primarily on the nitro group or the result of reduction potential. Is it too much of a strain on our imagination to assume that the harmful action of bacteria on the host may in part be the result of disturbance of definite chemical reactions? The example before us is reduction with special reference to the nitro group.

The reduction of p-nitro malachite green by various bacteria gives us a new indicator which enables the grouping of bacteria in certain broad classes. It also gives to bacteriology a new indicator that promises helpfulness in isolation and diagnosis. There is no parallelism between p-nitro malachite green and other commonly used tests, such as indol production, nitrate reduction, the neutral red test, or the manufacture of gelatinase, the gram stain or, in fact, to catalaze formation which has been advanced by McLeod and Gordon [5] as a basis for new classification of bacteria. Whether or not the specificity of the reaction is the result of a direct attack of bacteria on the nitro group or indirectly through reduction potential must be left to further investigation.

SUMMARY

A green dye, p-nitro malachite green, is reduced and changed to a red one, p-amino malachite green, by certain micro-organisms under prescribed conditions.

[4] Arch. f. ges. Phys., 1922, 196, p. 463.
[5] J. Path. & Bacteriol., 1923, 26, p. 326.

The reduction of the p-nitro group to p-amino may be interpreted as an index of the oxidation reduction potential of growing micro-organisms.

Thirteen different bacterial species were studied. When grown on plain broth, they all gave positive reactions, except B. alkaligenes, B. diphtheriae, and pneumococci.

In 10% horse serum, B. typhosus and meningococci were the only species studied which gave a pronounced reaction. Staphylococcus aureus and albus and Streptococcus hemolyticus gave a slight reaction (trace) in plain broth and in horse serum. B. coli caused a marked reduction in plain broth but only a slight reaction in the serum.

EXPERIMENTAL POLIOMYELITIS IN RABBITS

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The object of this work was to repeat certain experiments on poliomyelitis in rabbits. A number of workers have reported varying results. Some have found it difficult or impossible to inoculate rabbits and produce a picture resembling poliomyelitis; others assert that they were able to make successful inoculations and to pass the virus through a number of generations. All who were successful have found, however, that it was only in young rabbits that any effects were produced; that the results were variable and that the symptoms were always different from those observed in monkeys and in man.

The most recent work on experimental production of poliomyelitis in rabbits is that of Rosenau and Havens.[1] They passed poliomyelitis virus from a monkey through eight generations of rabbits. The virus showed no signs of dying out. It gave no evidence of becoming more virulent. The incubation period remained variable, and the percentage of positives did not increase. All inoculations were by no means successful, and there was great individual difference in susceptibility. Only 40% of the animals succumbed. These authors describe two types of symptoms. In one type, designated as the "progressive," there was paralysis of one extremity progressing until death occurred, resembling somewhat the type of disease found in monkeys and man. In the other group, designated as the "fulminating" type, the symptoms were more explosive in character with extreme weakness and prostration, death coming on within a few hours as a result of respiratory failure. The period of incubation was variable. It did not depend on the route of inoculation. The pathologic changes were different from those in monkey and man so far as microscopic examinations were concerned. "They lacked the distinctive features of the pathological picture of poliomyelitis in man and the monkey. Capillary congestion, punctate hemorrhages, degeneration of the motor cells, satellitosis and more or less cellular infiltration of the gray matter of the cord and medulla are found, but perivascular infiltration is absent and the infiltrating cells are not lymphocytic in character.' These authors feel, however, that they were dealing with a modified form of poliomyelitis; that the rabbit reacts differently to the virus than do monkeys and man. They raise the question, therefore, whether poliomyelitis may not occur in other animals in some hitherto unrecognized or unnoticed form.

Krause and Meinicke[2] and Lentz and Huntemüller[3] had previously reported the successful passage of poliomyelitis virus from rabbit to rabbit through suc-

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[1] Jour. Exper. Med., 1916, 23, p. 461.
[2] Deutsch. med. Wchnschr., 1909, 35, p. 1825; 1910, 36, p. 693
[3] Ztschr. f. Hyg. u. Infektionskrankh., 1910, 66, p. 481.

cessive generations. They found only slight lesions in the brain and spinal cord. These workers obtained better results by intravenous and intraperitoneal inoculations than by the intracerebral route.

Failure to inoculate rabbits successfully has been reported by Römer and Joseph,[4] Landsteiner and Levaditi.[5] Leiner and von Wisner[6] and by Flexner and Lewis.[7]

Interesting results were obtained by Marks[8] who was able to pass poliomyelitis virus through 7 generations of rabbits. The effects in rabbits, however, were not definite. Death occurred in some of these animals in from 7 to 11 days, with development of motor weakness and convulsions just preceding death. Monkeys inoculated with brain suspensions of these experimental rabbits developed similar symptoms. The pathologic changes noted in the rabbits were not characteristic, the only findings being hyperemia of the brain. As Marks states: "The disease thus produced in rabbits cannot be recognized as poliomyelitis." He finds further that "not all strains of the virus can be transmitted successfully to even a small fraction of all varieties of domesticated rabbits."

A 5% unfiltered suspension of poliomyelitis virus was used. This had been obtained from the Rockefeller Institute. Inoculations were made intracerebrally, intraperitoneally and intravenously. The intracerebral route was the one most frequently employed. In some animals the intracerebral route was combined with the intravenous or with the intraperitoneal route. Only young rabbits were used; the average age was 6 weeks.

First Passage.—Ten rabbits, 344 to 353, were inoculated intracerebrally with from 0.25 c c. to 2 c c. At the same time monkey X was inoculated intracerebrally with 0.6 c c. of the same virus. Only one rabbit, 348, developed typical symptoms (6th day); the animal was chloroformed on the 13th day. One rabbit was discharged after 10 weeks, and the remaining rabbits died after from 24 hours to 1 month. Monkey X, used as a control, developed typical symptoms of poliomyelitis on the 5th day and died on the 9th day. Examination showed the changes characteristic of poliomyelitis.

Second Passage.—Pooled brain suspension from 2 rabbits (348 and 350) of the first passage was inoculated into 2 rabbits, 358 and 359, both intracerebrally and intravenously. One of the rabbits (348) furnishing this virus, it will be recalled, was the one which had developed typical, progressive, paralytic symptoms, and the other (350) had been found dead after 7 days.

Brain suspension of monkey X was also inoculated in a second series of 4 rabbits, 354 to 357, (2d passage of virus). The doses varied from 1 c c. to 1.5 c c. The intracerebral route alone was used in this group. None of these animals developed any symptoms.

Third Passage.—The pooled brain suspension from rabbits 355 and 356 was inoculated into a series of 4 rabbits (372 to 375). One of the rabbits (374) developed paralytic symptoms on the 14th day. The symptoms were similar

[4] Münch. med. Wchnschr., 1916, 57, p. 2685.
[5] Compt. rend. Soc. de biol., 1909, 67, p. 787.
[6] Wien. klin. Wchnschr., 1909, 22, p. 1698.
[7] Jour. Exper. Med., 1910, 12, p. 227.
[8] Ibid., 1911, 14, p. 116.

to those observed in rabbit 348 of the 1st passage. Suspension from the brain and cord of rabbit 356 was also inoculated in a 2d set of rabbits (376 and 377), both intracerebrally and intraperitoneally in the one, and intracerebrally alone in the other. The doses and results are shown in the chart. No symptoms developed in these animals.

Fourth Passage.—A suspension from the brain of rabbit 374 of the 3rd passage of virus was next inoculated in a series of 10 rabbits (378 to 387). The doses from the intracerebral inoculations varied from 0.25 c c. to 0.5 c c., and all rabbits of this series except one were also inoculated intraperitoneally with 2 c c. of the suspension. All of these rabbits died in from 15 minutes to 4 days. No symptom developed.

Fifth Passage.—A pooled brain, and cord suspension from rabbits 378, 379, 382, 383, and 387 was inoculated in a series of 4 monkeys (93, 52, 53 and 31) and 6 rabbits (394 to 399). The monkeys were given 1 c c. of suspension intracerebrally and from 3 to 20 c c. intraperitoneally. The rabbits were given 0.4 and 0.5 intracerebrally and 5 c c. intraperitoneally. In none of the animals of this series did any symptoms develop. The virus had apparently died out.

Pathologic Changes.—Tissues of rabbits 350, 351, and 353, examined by Christine I. Gruggel, showed a slight amount of neurophagocytosis of the ganglion cells and some cell degeneration. There was no evidence of round cell perivascular infiltration. As the degeneration of the ganglion cells might be due to other causes, there was no conclusive evidence of poliomyelitis, but it cannot be excluded.

In rabbits 344, 345, 348, 373, 374, 375, 377, 395, the meninges were normal; in the gray matter occasional ganglion cells showed cytolysis and neurophagocytosis, the later being mononuclear. There was no cellular infiltration or evidence of poliomyelitis.

Sections of monkey X showed a round cell infiltration of the meninges following the vesels into the gray matter of the anterior horns. In places were patches of exudate in which was degeneration of ganglion cells, with neurophagocytosis—a typical picture of poliomyelitis.

DISCUSSION

In only 2 rabbits did symptoms occur that were at all comparable to those in poliomyelitis of monkeys and of man or that corresponded with those described by Rosenau and Havens [1] in their "progressive paralytic" type. One of these rabbits (348) of the 1st passage of virus, developed symptoms after an incubation period of 6 days. There was weakness of the posterior extremities, which were held wide apart. There was definite paralysis of the left foreleg and paresis of the right foreleg. This animal was chloroformed on the 13th day. Necropsy did not show any definite lesions, and the pathologic examinations showed only some cytolysis of the ganglion cells and mononuclear neurophagocytosis. There was no cellular infiltration. Cultures to determine the presence of aerobic and anaerobic micro-organisms were all negative. Rabbit 374 of the 3rd passage of virus developed similar

symptoms on the 14th day. This rabbit had been inoculated with virus passed through monkey X, used as a control in the first passage; the changes were the same as in rabbit 348.

Of the remaining 26 rabbits which succumbed, 5 died within 24 hours or less. These must be left out of consideration, as death may have been due to injury or shock. In the case of 3 rabbits dying almost immediately, anaphylactic shock due to a foreign protein may have been the cause. In the case of 21 rabbits surviving the first 24 hours and living in some cases as long as a month, it is impossible to determine the cause. Some of them exhibited the symptoms described by Rosenau and Havens as of the "fulminating" type. Some of them were found dead without any symptoms having been noted. In the necropsis and examinations of tissue sections of these animals, no changes characetristic of poliomyelitis were found; at least there was an absence of such changes as are found in poliomyelitis of monkeys and man. The dose and the route of inoculation does not seem to have caused any difference in the effect. The period of incubation was variable in all passages. There was apparently no change in the virulence of the virus.

The question of anaphylactic shock was carefully considered in carrying out these experiments. A series of 6 rabbits, 388 to 393, were inoculated with 5% suspension of normal rabbit's brain. None of these animals developed any symptoms, and they were discharged after a period of 2 months. This may, however, be merely a coincidence. It may be that none of the animals in this small group of controls were sensitive to the brain protein of the particular group of animals used.

It is difficult to make any definite deductions from the results of the foregoing investigation. If the rabbits exhibiting the "fulminating" type of symptoms died as a result of poliomyelitis virus, we should be forced to accept the suggested hypothesis of Rosenau and Havens that in some species of animals, poliomyelitis develops or assumes a type of disease hitherto unrecognized or unnoticed. Even in the 2 rabbits which developed symptoms similar to those occurring in poliomyelitis of monkeys and of man, the histologic changes were not characteristic. As, however, in both types of effects certain histologic changes were present, it cannot be denied absolutely that they were caused by poliomyelitis virus. The changes present, although different from those found in monkeys and man, may nevertheless be the changes produced by poliomyelitis in rabbits. The fact that one of the rabbits developed typical symptoms from virus that had been passed through a monkey and one generation of rabbits, would certainly suggest such a

possibility. The symptoms and pathologic changes were the same as in the case of the rabbit of the first passage inoculated directly with fixed virus.

SUMMARY

In the experiments on young rabbits, symptoms resembling poliomyelitis were observed in a small number. A larger number of rabbits died after varying periods of incubation, but without showing characteristic symptoms of poliomyelitis. The virus did not show any increasing virulence by passage through rabbits. In one series of animals, the virus apparently died out after one passage. In the other series, it apparently died out after passage through a monkey and 3 sets of rabbits. Brain suspension from this 3rd passage through rabbits, inoculated in both monkeys and rabbits failed to have any effect. In the case of the animals which died, the factor of infection was carefully controlled by cultures; also the possibility of anaphylactic shock due to a foreign protein was taken into account by inoculating a series of young rabbits with normal rabbit brain suspension. All of these controls were negative. It cannot be said that we have been able successfully to inoculate rabbits with poliomyelitis.

SPONTANEOUS RECOVERY OF RABBIT FROM EXPERIMENTAL RABIES

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Subdural inoculations of rabbits with appropriate doses of fixed rabic virus are generally followed by the development of rabies. Relatively few cases of nondevelopment of the disease after such injections have been reported.[1] The spontaneous recovery of rabbits after the establishment of typical symptoms subsequent to subdural injection of fixed virus is still rarer. Our search of the literature reveals only one reported case, that of Remlinger.[2] Our own experience covering a number of years and comprising the inoculation of hundreds of rabbits leads us to disagree with the statement that recovery of rabbits showing rabid symptoms after such inoculation is common, even though the subdural injection has been made with minute amounts of active fixed virus. We have seen only one case of such recovery, and believe it so uncommon as to be worthy of report.

On Jan. 6, 1922, during the course of preparation of a batch of fixed virus for desiccation and subsequent use as a prophylactic vaccine, 6 rabbits ranging in weight between 2,000 and 2,400 gm. were injected directly into the ventricle through a trephine opening in the skull with 500 minimal infective doses[3] of fixed desiccated virus. On the 7th day, 5 of the rabbits began to show signs of rabic paralysis and died, 3 on the 8th day and 2 on the 9th day. The 6th rabbit, a female weighing 2,140 gm. at the timeof inoculation, although droopy and manifestly sick on the 7th day, did not show any beginning paralytic symptoms before the 11th day after inoculation. Then there appeared involvement of the hind quarters, and although the animal could still maintain itself on its four extremities, its gait was unsteady with marked spasticity of the limbs; its appetite was fair. On the 14th day, the condition was aggravated. The animal could barely stand, and when it did assume the normal standing position, it quickly fell. Its back was arched and its head drooped, evidently as a result of paralysis of the muscles of the neck. It made feeble attempts at this time to take food. On the 15th day the animal was lying on its side in the cage, and occasionally made feeble efforts at moving its hind quarters; the respiratory center was involved, respiration being short, rapid, irregular. On the afternoon of the 16th day, much of our surprise, there was marked amelioration of the symptoms. Respiration was slow and regular; the animal moved its head from

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[1] Remlinger: Ann de l'Inst. Pasteur, 1903, 17, p. 843; Comp. rend. Soc. de biol., 1918, 81, p. 162. Viala, J.: Ann. de l'Inst. Pasteur, 1912, 26, p. 239.

[2] Ibid., 1919, 33, p. 735.

[3] D'Aunoy, R.: Jour. Infect. Dis., 1922, 30, p. 347.

side to side and made attempts at eating grass. It appeared brighter, and there were apparent feeble movements of the hind quarters. Saliva was secured at 8 p. m. on this day and injected subdurally into 2 rabbits. On the 11th day, the animal would stand at times, but seemed to have lost the power of coordinated movements. Its appetite was fair, its head still being drooped. On the 18th day, the paralysis was rapidly disappearing; the animal was up on its fore quarters, but its gait was unsteady, and it frequently staggered. Its head was still somewhat drooped. Its appetite was fair. The 19th to 21st day brought rapid recovery. At times there was a swaying or staggering gait with a tendency to move about in a circle, which persisted for 3 or 4 days. The appetite was good, with an apparent gain of weight. On the 25th day, the animal was normal. On the 40th day, saliva was again secured and injected subdurally into two

TABLE 1

EFFECTS OF SERUM ON ONSET AND COURSE OF DISEASE

(Intracranial injections of virus-serum mixtures kept at 37.5 C. for one hour)

Fixed Rabic Virus	Undiluted Serum	Appearance of First Symptoms	Death
1 M.I.D.	0.5 c c.	8th Day	11th Day
1 M.I.D.	1 c c.	9th Day	12th Day
1 M.I.D.	2 c c.	14th Day	19th Day
1 M.I.D.	3 c c.	10th Day	11th Day

TABLE 2

SIMULTANEOUS INTRACRANIAL INJECTION OF VIRUS, AND INTRAPERITONEAL INJECTION OF SERUM

Fixed Rabic Virus	Undiluted Serum	Appearance of First Symptoms	Death
1 M.I.D.	2 c c.	8th Day	11th Day
1 M.I.D.	5 c c.	9th Day	12th Day
1 M.I.D.	8 c c.	9th Day	11th Day
1 M.I.D.	10 c c.	8th Day	11th Day

full-grown rabbits. At the same time subdural injection of 1,000 minimal infective doses of fixed virus was made into the apparently fully recovered animal, with similar injection into 2 control animals. On the 40th day, the animal was normal. Controls previously injected were dead, with typical symptoms of rabies. On the 60th day, the animal was still normal.

The rabbits injected subdurally with the saliva secured on the 16th day all developed typical rabies. Emulsions from their brain regularly caused death in subsequent injections. Smears from Ammon's horn and the cerebellum as well as brain sections stained by Mallory's method showed Negri bodies in all the animals of the series. In these early passage animals, however, typical large intracellular Negri bodies were rarely encountered; here the bodies were few in number, small and often atypical in morphology. This is in keeping with the observations of Cruikshank and Wright,[4] and Phillips, Berry and Snook.[5]

The rabbits injected with saliva secured on the 40th day failed to developed rabies. That they were not naturally resistant, was demonstrated by their subsequent infection with one minimal infective dose of desiccation fixed virus.

[4] Indian Jour. Med. Res., 1914, p. 562.

[5] Jour. Infect. Dis., 1921, 29, p. 97.

When it appeared that there was a possibility of the animal recovering, blood was obtained by cardiopuncture in order to study its possible rabicidal properties. This puncture was repeated on the 90th day after inoculation with the same object. Clear serum was used for all the tests, and an attempt was made to determine the amount of serum necessary to neutralize one minimal infective dose of desicated fixed virus. Table 1 and 2, which express the details of the experiment with the serum obtained at the first bleeding, show no appreciable antirabicidal action. In one animal of the intracranial series, the onset of the disease was late and death somewhat prolonged, but even though we take into consideration the sharply limited zone of action of so-called antirabicidal serum, seen especially after intensive immunization, we are unable to ascribe in this case any appreciable protective powers to the blood serum of this recovered animal. With serum obtained at the second bleeding, no suggestive antirabicidal action could be noted.

SUMMARY

A case of spontaneous recovery of a rabbit from rabies after full development of symptoms following intracerebral injection of fixed desiccated virus is reported.

Saliva from this animal obtained 16 days after inoculation when injected intracerebrally into rabbits produced typical rabic symptoms with Negri bodies in brain sections. Forty days after inoculation the animal's saliva was no longer infectious.

Blood serum secured 17 and 90 days after injection showed no appreciable antirabicidal action.

SPONTANEOUS ENCEPHALITIS IN RABBITS

ONE PLATE

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In studying the brains of rabbits infected with the virus of herpes simplex, it was found that a large proportion of the animals with which we were working had chronic focal lesions within the brain and spinal cord and an exudate in the meninges consisting entirely of mononuclear cells. These lesions were attributed to a latent or pre-existing infectious disease. An apparently identical disease of the brain of rabbits has been described by Bull,[1] Oliver,[2] and more recently by Twort and Archer.[3] Bull and Oliver found the lesions only in the central nervous system, while Twort connected the cerebral changes with similar foci in the kidneys, which he attributed to an identical etiologic agency. These observers failed to find organisms associated with the lesions, and Twort, on the questionable ground that the cellular reaction consisted of cells of a mononuclear type only, assumed the etiologic agent to be a filtrable virus.

In an examination of the brains of 30 rabbits bought from local dealers, I have found definite focal lesions of apparently the same disease in 10 instances; and in a majority of the 30 animals there was an exudate in the meninges more or less prominent, consisting of mononuclear cells. The disease has a characteristic appearance as exhibited in sections of the brain. The lesions are focal in character and may be irregularly distributed throughout the cerebral substance from the olfactory lobes into the spinal cord; and from 1 to 25 foci may appear in a single cross section of the brain. Their size is fairly uniform and approximates that of a miliary tubercle. They do not appear to be more numerous in any particular portion of the brain. It is usually evident that each focus is related to a small blood vessel to one side of which it is situated, this vessel and others in the neighborhood generally presenting a mantle of small mononuclear cells. The lesions themselves consist of a circumscribed round or oval compact aggregation of large

Received for publication, Dec. 10, 1923.

[1] Jour. Exper. Med., 1917, 25, p. 557.
[2] Jour. Infect. Dis., 1922, 30, p. 91.
[3] Lancet, 1923, 204, p. 1102.

mononuclear cells with irregular nuclei and often with abundant cytoplasm. Small mononuclear cells are also present. Not infrequently there is necrosis at the center of the cellular accumulation. In the meninges, in addition to small and large mononuclear cells of the lymphocytic series, there are usually numerous plasma cells, but polymorphonuclear leukocytes are absent.

In the brains of 32 rabbits in the series examined, lesions of this character were particularly numerous and were associated with more necrosis in the center than is ordinarily found. Sections from these brains stained for bacteria disclosed numerous large organisms in the necrotic centers of many of the focal lesions. These organisms are bacillary in shape with rounded or conical ends and measure about 4 microns in length and 1 micron in breadth. They are in groups or irregularly distributed within the necrotic portion of the lesion and some are degenerated within the large mononuclear cells.

A further opportunity to study this organism and the disease it produces was accidentally afforded by the following experiment:

On Feb. 26, 1923, 4 c c. of filtered washings from the nose and throat of a patient presenting symptoms of epidemic encephalitis were injected slowly into the right side of the brain of an adult white female rabbit. A week later the animal tended to hold its head to the left and showed certain peculiarities of behavior which were attributed to injury of cerebral tissue by the injection of such a large quantity of fluid. Three weeks after the injection she gave birth to 4 young, all white. These seemed to be normal and developed normally for 3 weeks. They were kept in a cage with their mother, isolated from other animals. When 3 weeks old, 2 of the young became drowsy, and they would sit quietly in the cage and nod. When quiet, the eyes would close and the head would sway slowly to one side. When aroused, they seemed active and quite normal, but they became poorly nourished and the hair took on a dirty yellowish color. These symptoms continued and increased in severity. The other 2 young showed similar though much milder indications of disease. On April 28, one of the litter which showed most marked symptoms died, and the other showing evident symptoms was killed. A portion of the brain of each was inoculated into each of two half-grown rabbits. These animals were drowsy for 3 weeks but afterward showed no noticeable symptoms.

The brains of the two young rabbits in sections presented numerous focal lesions and a meningitis in all respects similar to that found in the adult rabbits. In addition, earlier stages in the development of the lesion were present. Numerous micro-organisms like those in the lesions of the adult rabbits were observed. They occurred in groups in the center of a cellular focus at times appearing as if they were incorporated within the cytoplasm of an enlarged cell, or they were irregularly scattered about between the inflammatory cells. Occasionally a group was seen in a part of the brain free from cellular exudate. Early lesions were found in which the organisms seemed to be within the wall of a capillary. The characteristics of these organisms are sufficient to identify them with those present in the brains of the adults.

On April 30, 2 young white and 2 young gray rabbits, 1 month old, were placed in the same cage with the mother of the 4 infected young. Two weeks

later the white rabbits became drowsy and exhibited a slow movement of the head to one side then to the other; the gray rabbits remained normal. Two weeks after the appearance of these symptoms, the 2 white animals were killed and sections from different organs and tissues, including the central nervous system, were studied histologically. Focal lesions were found in the brain, and similar lesions were present in abundance in the kidneys and lungs. The lungs presented no noticeable change in gross, but the kidneys exhibited a mottling with pin point gray dots, especially numerous in the medulla. Microorganisms identical morphologically with those in cerebral lesions were found in the lungs and in the kidneys. In the lungs, they occurred in groups in the alveolar wall with a focal exudate of mononuclear cells. In the kidneys, they were present not only in collecting tubules of the medulla within the focal lesions, but also within the cytoplasm of the epithelial cells of these tubules. In both these organs, the organisms were found in only a few foci, although the lesions were numerous. In the kidneys and lungs, as in the brain, the lesions were vascular in distribution. No similar changes were noted in the other organs and tissues.

The morphologic details of the organisms are the same in the cerebral lesions of the young rabbits and in the adults. There is sometimes a tendency for them to stain more deeply in the center than at the poles, again the poles are stained more prominently; and there may be unstained areas somewhat like vacuoles within the parasites. The organisms are gram-positive and slightly acid fast. They are easily demonstrable in preparations stained by Gram's method or with carbol-anilin-fuchsin, differentiated with formaldehyde and counter-stained with picric acid after fixation in Zenker's fluid.[4]

Cultures of the brains of these animals have remained sterile, and animal inoculation has up to the present yielded questionable results. The microscopic appearance of the cerebral lesions of this disease in the spontaneously infected adults and in the young contracting the infection in the laboratory, presumably from the mother which was inoculated intracerebrally with the filtered washings from a suspected case of encephalitis, are identical with those described by Bull and by Oliver. They have also a striking similarity to the illustrations of some of the lesions found by Loewe and his co-workers[5] in rabbits' brains in their experimental work with material from cases of human encephalitis, and to those more recently demonstrated by Kling, Davide and Liljenquist,[6] in brains of these animals inoculated with similar material.

The lesions in the kidneys of young infected rabbits correspond to those described by Twort and Archer as produced experimentally by inoculation with their "virus."

[6] Compt. rend. Soc. de biol., 1921, 84, p. 521.
[4] Goodpasture, E. W., and Burnett, F. L.: U. S. Naval Med. Bull., 1919, 13, p. 177.
[5] Jour. Am. Med. Assn., 1919, 73, p. 1056; 1920, 74, p. 1373.

There seems to be little doubt that these lesions represent a spontaneous disease of rabbits, widespread in its distribution and present as a latent infection in a large proportion of these animals used in the laboratory both in this country and abroad. McCartney,[7] at the Rockefeller Institute, in a series of 300 rabbits, found the lesions in the brain in about one-half this number.

The micro-organisms which I have found are similar in morphology, staining qualities, relation to the lesion, and distribution, to the micro-organisms described by Wright and Craighead[8] as the cause of "infectious motor paralysis in young rabbits." In an attempt to infect young rabbits with the virus of human infantile paralysis, these authors observed incidentally and "presumably accidentally" a paralytic disease associated with an organism having characteristics similar to those which I have described. The disease was transmissible by direct contact, presumably from eating food contaminated with the urine of infected rabbits, which was shown to contain the organisms, and by direct inoculation of infected material into the brain. In their experience, however, the organisms, while widely distributed in the organs of an infected animal, caused especially a destruction of nerve cells of the spinal cord, which explained the clinical symptoms. They were unable to cultivate the organism and thought it probably represented an intermediate stage in the life history of some protozoan parasite. Wright and Craighead, however, did not associate this disease with the prevalent infection so commonly found in the brains of adult rabbits, but a comparison of the organism which I have observed with their illustrations leaves little doubt that they are the same. The clinical course of the infection which they induced in young rabbits differs, however, in that no paralytic symptoms were noted in my animals.

The similarity of the lesions of the spontaneous disease observed in our animals has convinced me that they are identical with those described by Bull, Oliver, Twort and Archer, McCartney, and by Wright and Craighead; and that this widespread disease of rabbits is caused by a single infectious agent first described by the last authors, but not recognized by them as the probable cause of a common endemic disease in these animals. Wright and Craighead described a generalized infection affecting particularly the central nervous system and the kidneys. Twort and Archer first drew attention to the association of

[7] Quoted by Flexner, Jour. Am. Med. Assn., 1923, 81, p. 1785.
[8] Jour. Exper. Med., 1922, 36, p. 135.

renal involvement and lesions in the central nervous system in adult rabbits affected apparently with the same disease, but attributed by them to a filtrable virus. Finally a demonstration of morphologically identical organisms in the brain and kidneys of rabbits with both conditions lends strong evidence of their identity in etiology.

In this disease it would appear we must recognize a generalized infection, the lesions of which are vascular in distribution affecting chiefly the brain and kidneys and less conspicuously the spinal cord and lungs.. The nature of the invading micro-organism is uncertain, but it may be as suggested by Wright and Craighead, an intermediate stage in the life history of a protozoan parasite, although in many ways it resembles a bacterium. The disease is highly contagious and may be spread by food contaminated with the urine of infected persons. Ordinarily it is a chronic disease with low mortality, except perhaps with certain strains, particularly in young rabbits; and it seems to produce little immunity, but persists as a latent infection into adult life causing few or no symptoms. In our experience, white rabbits appear to be more susceptible to infection than gray ones, and it is believed the disease may occasionally be recognized in mildly active form in adult white rabbits by the symptom of a slow lateral movement of the head from side to side when they are otherwise quiet.

The practical significance of this disease as a source of confusion in experimental work with rabbits, especially in connection with attempts to transmit to these animals the virus of such human diseases as epidemic poliomyelitis, epidemic encephalitis and herpes, has recently been pointed out by Flexner,[9] and it is of utmost importance that the pathologic changes associated with it be recognized, so that due evaluation may be given them in determining the results of any experimental study of infections of the rabbit's brain, lungs or kidneys.

SUMMARY

Large gram-positive bacillary organisms have been demonstrated in the cerebral lesions of spontaneous encephalitis of rabbits.

The cerebral lesions are vascular in distribution and are the manifestation in the brain of a generalized infection which may produce similar lesions in the lungs and kidneys in which morphologically identical organisms have been found.*

[9] Jour. Am. Med. Assn., 1923, 81, p. 1785.

* Since this paper was submitted for publication I have seen the papers by Levaditi and Nicolau (Compt. rend. Soc. de biol., 1923, 79, p. 984, 1157), describing the micro-organism named by them, "Encephalitozoon cuniculi," which they have demonstrated in the cerebral lesions of this disease in rabbits. I regard the micro-organism which I have described as the same parasite.

PLATE 1

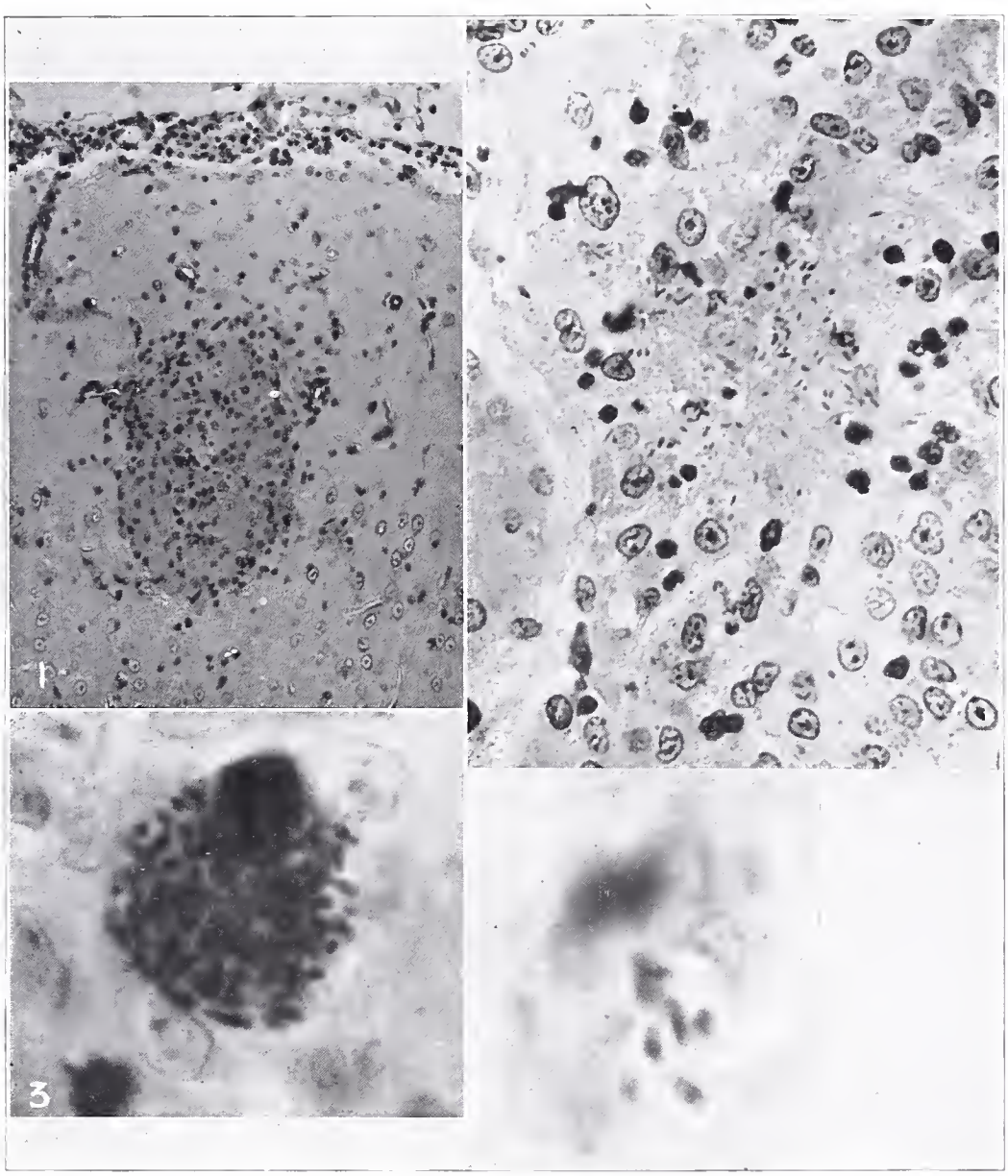

Fig. 1.—Focal lesion in frontal lobe with cellular exudate in meninges in an adult rabbit.

Fig. 2.—Micro-organisms in central necrotic area of encephalitic focus in adult rabbit; × 1,000.

Fig. 3.—Group of micro-organisms in center of encephalitic focus in young rabbit; × 1,500.

Fig. 4.—Micro-organisms in wall of capillary in young rabbit's brain; no cellular exudate; centers of organism stained more deeply than poles; × 2,000.

FURTHER OBSERVATIONS ON LENS PRECIPITINS

ANTIGENIC PROPERTIES OF ALPHA AND BETA CRYSTALLINS

LUDVIG HEKTOEN AND KAMIL SCHULHOF

From the John McCormick Institute for Infectious Diseases, Chicago

1. SPECIFIC PRECIPITINS FOR ALPHA AND BETA CRYSTALLINS

The precipitate was dissolved by adding a little ammonia, then diluted with water and then reprecipitated as described several times. The filtrate containing the beta crystallin and albumin was dialyzed and acetic acid added again to remove any remaining alpha crystallin. Finally, 0.85% of sodium chlorid was added to both solutions. Preparations of beta crystallin which contained lens albumin did not seem to act differently from those in which the albumin was removed by half saturation with ammonium sulphate. We did not succeed in obtaining any precipitins by injecting rabbits with the albumin from beef lens, perhaps because of the small amount injected. Rabbits were injected

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[1] Ztschr. f. physiol. Chemie., 1894, 18, p. 61.
[2] Arch. f. Ophth., 1909, 7, p. 165; 1922, 199, p. 463.

CORRECTION

Dr. Martin Kristensen, of Copenhagen, whose monograph on the Pfeiffer bacillus was referred to several times in a recent article in this Journal (Jordan, E. O., and Reith, A. F.: Jour. Infect. Dis., 1924, 34, p. 239), writes that, owing to an error in translating or printing his manuscript, he was made to affirm the frequent presence of indol formers among hemolytic Pfeiffer bacilli. His statement on this point should read: "Eighteen strains [hemolytic, hemoglobinophilic bacilli] were examined for indol formation, all with negative results." His observations were hence not at variance with those recorded in this Journal.

FURTHER OBSERVATIONS ON LENS PRECIPITINS

ANTIGENIC PROPERTIES OF ALPHA AND BETA CRYSTALLINS

LUDVIG HEKTOEN AND KAMIL SCHULHOF

From the John McCormick Institute for Infectious Diseases, Chicago

1. SPECIFIC PRECIPITINS FOR ALPHA AND BETA CRYSTALLINS

In 1830, Berzelius isolated a globulin from the lens, and in 1842 Simon showed that the lens contains more than one protein substance. In 1894, Mörner [1] isolated from beef lens alpha crystallin, beta crystallin, an albumin, and an insoluble albuminoid substance. The studies by Jess [2] deal especially with the constitution of these proteins and their proportions in the lens. Jess finds the proportion of alpha to beta in youth is 82 : 18; in aged persons, 41 : 59; and in cataractous lenses, 25 : 75. In cataracts the absolute amounts of both are reduced. The outer parts of the lens contain more alpha crystallin, the inner more beta. The question arises whether alpha and beta crystallins differ in their precipitin reactions. Accordingly, crystallins were prepared from beef, swine, and other lenses in the following manner: When necessary the capsule was removed, the lens substance crushed with quartz sand and then thoroughly shaken in water. This extract was centrifugated and filtered through paper to secure a clear fluid. Alpha crystallin was thrown down by adding a 1% solution of acetic acid, enough to give a concentration in the lens extract of from 0.02 to 0.04%. The carbon dioxide method seemed to give less satisfactory results. The precipitate was dissolved by adding a little ammonia, then diluted with water and then reprecipitated as described several times. The filtrate containing the beta crystallin and albumin was dialyzed and acetic acid added again to remove any remaining alpha crystallin. Finally, 0.85% of sodium chlorid was added to both solutions. Preparations of beta crystallin which contained lens albumin did not seem to act differently from those in which the albumin was removed by half saturation with ammonium sulphate. We did not succeed in obtaining any precipitins by injecting rabbits with the albumin from beef lens, perhaps because of the small amount injected. Rabbits were injected

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[1] Ztschr. f. physiol. Chemie., 1894, 18, p. 61.
[2] Arch. f. Ophth., 1909, 7, p. 165; 1922, 109, p. 463.

intravenously with solutions of lens, of alpha crystallin or of beta crystallin, and the precipitin reactions of the serum studied in the manner described elsewhere.[3] For immunizing rabbits with either crystallin, 1 or 2% and in some cases weaker solutions were injected into the ear vein in increasing quantities—2, 4, 6, 8, 10 c c.—at 3-day intervals. As a rule, the serum had a fairly high precipitin titer on the 4th or 5th day after the last injection. The results of the precipitin tests establish that alpha and beta crystallin of the bovine lens and also of other species are precipitinogenically distinct, the serum of rabbits injected with beef alpha crystallin reacting with alpha only and not with beta, and

TABLE 1

PRECIPITINS FOR BEEF CRYSTALLINS

Antigens	Precipitin Serum for Alpha Crystallin			Precipitin Serum for Beta Crystallin		
	Original	After Absorption with Alpha Crystallin	After Absorption with Beta Crystallin	Original	After Absorption with Alpha Crystallin	After Absorption with Beta Crystallin
Beef lens	128,000	0	80,000	500,000	320,000	0
Beef alpha crystallin	1,600	0	800	100	0	0
Beef Beta crystallin	0	0	0	6,400	3,200	0
Rabbit lens	100,000	0	16,000	100,000	32,000	0
Human lens, normal	3,200	0	1,600	100,000	50,000	0
Human lens, cataractous	3,200	0	1,600	12,800	6,400	0
Swine lens	32,000			32,000		
Swine alpha crystallin	16,000					
Swine beta crystallin	1,000			8,000		

vice-versa (table 1). Absorption experiments confirm this result, beef alpha crystallin removing the alpha precipitin but not the beta, and vice-versa. The precipitins for beef crystallins react also with the crystallins of other species as well as with solutions of the whole lens—beef, rabbit, human (normal and cataractous), monkey, swine and menhaden lens—thus indicating that the organ-specificness of the lens depends largely if not wholly on the presence in the lens, at least of the species mentioned, of alpha and beta crystallins. Table 1 shows that each crystallin removed from the homologous antiserum its power to react with solutions of the whole lens, which no doubt means that the crystallins exist as suc hin the lens. It may be stated heer that solutions of whole lens (beef), of beef alpha or beta crystallin do not

[3] Hektoen, Ludvig: Specific Precipitin Reaction of Normal and Cataractous Lens, Jour. Infect. Dis., 1922, 30, p. 40.

appear to react with precipitin serums for the albumin or globulin of beef blood serum.

Analogous results have been obtained in the case of swine, sheep, dog, and cataractous human lenses and their crystallins, but it proved much more difficult to secure these crystallins in pure form. In all cases, however, the alpha precipitin serums contained a great deal more precipitin for alpha crystallin than for beta crystallin, and vice-versa, indicating that in each case the antigen contained a relatively small amount of the undesired crystallin, a deduction that was strengthened by the results of tests of the antigens with anti-alpha and antibeta precipitin serums. With rabbit crystallins, we obtained in one instance a strictly specific result, the alpha and beta precipitins in that case not giving any cross reactions (table 2) with the rabbit crystallins. This

TABLE 2

PRECIPITINS FOR RABBIT CRYSTALLINS

Antigens	Precipitin Serum for Alpha Crystallin	Precipitin Serum for Beta Crystallin
Rabbit lens	960	240
Rabbit alpha crystallin	4,400	0
Rabbit beta crystallin	0	10,000

result is referred to again in the next part dealing with the formation of precipitins by rabbits injected with rabbit lens.

The figures in the tables giving the highest dilutions of lenses in which specific reactions occurred are based on the weight of the fresh lens dissolved in salt solution. As the water content of the lens no doubt varies, particularly in different species, and as no attempt was made to determine the amount of insoluble residue in each case, the figures must be regarded as merely approximate. In the case of the solutions of alpha and beta crystallins, however, the figures represent accurate dilutions.

2. PRECIPITIN FORMATION BY RABBITS IN RESPONSE TO INJECTIONS OF RABBITS LENS AND CRYSTALLINS

In a previous paper [3] it was pointed out that under ordinary circumstances rabbits were found to produce lens precipitins in response to injections of lens of other species only, and not in response to the injection of rabbit lens. Rabbits previously injected with foreign lens, that is, rabbits rendered allergic with respect to lens, may respond to the injection of rabbit lens with production of lens precipitins. Further

experiments along this line have confirmed this statement, and chart 1 is presented by way of illustration. It concerns a rabbit that on the 25th day after the last injection of a solution of human cataractous lens received intravenously 4 c c. of a 10% solution of rabbit lens which had been passed through a Berkefeld filter. As shown in the chart, there resulted a new production of precipitins for beef and cataractous lens and also for rabbit lens.

That the resistance of normal rabbits to the antigenic effect of rabbit lens may not be overcome in other ways than the one followed in this experiment is, of course, not at all excluded. Thus the intravenous injection of solutions of rabbit crystallins in normal rabbits may call forth precipitins for the lens and the crystallins of the rabbit and of

TABLE 3

PRECIPITIN REACTIONS OF FISH LENS

Antigens	Serum Against Menhaden Lens	Serum Against Cataractous Human Lens	Serum Against Beef Lens
Menhaden lens (Brevoortia tyrannus)	24,000	200	200
Mackerel lens (Scomber scombrus)	24,000	200	200
Butterfish lens (Stromateus triacanthus)	24,000	200	200
Bonito lens (Sarda sarda)	24,000	200	100
Sculp lens (Cottus scorpicus or aeneus)	24,000	80	400
Squeteague lens (Cyonoscion regalis)	24,000	80	500
Flounder lens (Pleuronectes platersa)	24,000	200	200
Beef lens	2,400	100,000	160,000
Beef alpha crystallin	200	400	800
Beef beta crystallin	800	400	1,600
Rabbit lens	6,400	20,000	16,000
Human lens, normal	3,200	100,000	40,000
Human lens, cataractous	1,600	100,000	24,000
Swine lens	3,200	12,000	16,000

other species as well. In this case, 4 c c. of a 1 : 1,100 solution of alpha crystallin were injected intravenously each day for 3 days; after an interval of 6 days, a series of 3 daily injections of 6 c c. was given; and after another 6-day interval, 3 daily injections of 8 c c. were given.

Injections of rabbit beta crystallin were made according to the same general plan, but in this case the strength of the solution was 1 : 10,000. The rabbits were bled on the 4th day after the last injection, when the serum was found to have a considerable precipitin strength (table 2). This result in immunization with homologous antigens invites further experiments. We are not blind to the possibility that the results with rabbit crystallins may have been influenced by changes in the antigens caused by the purification.

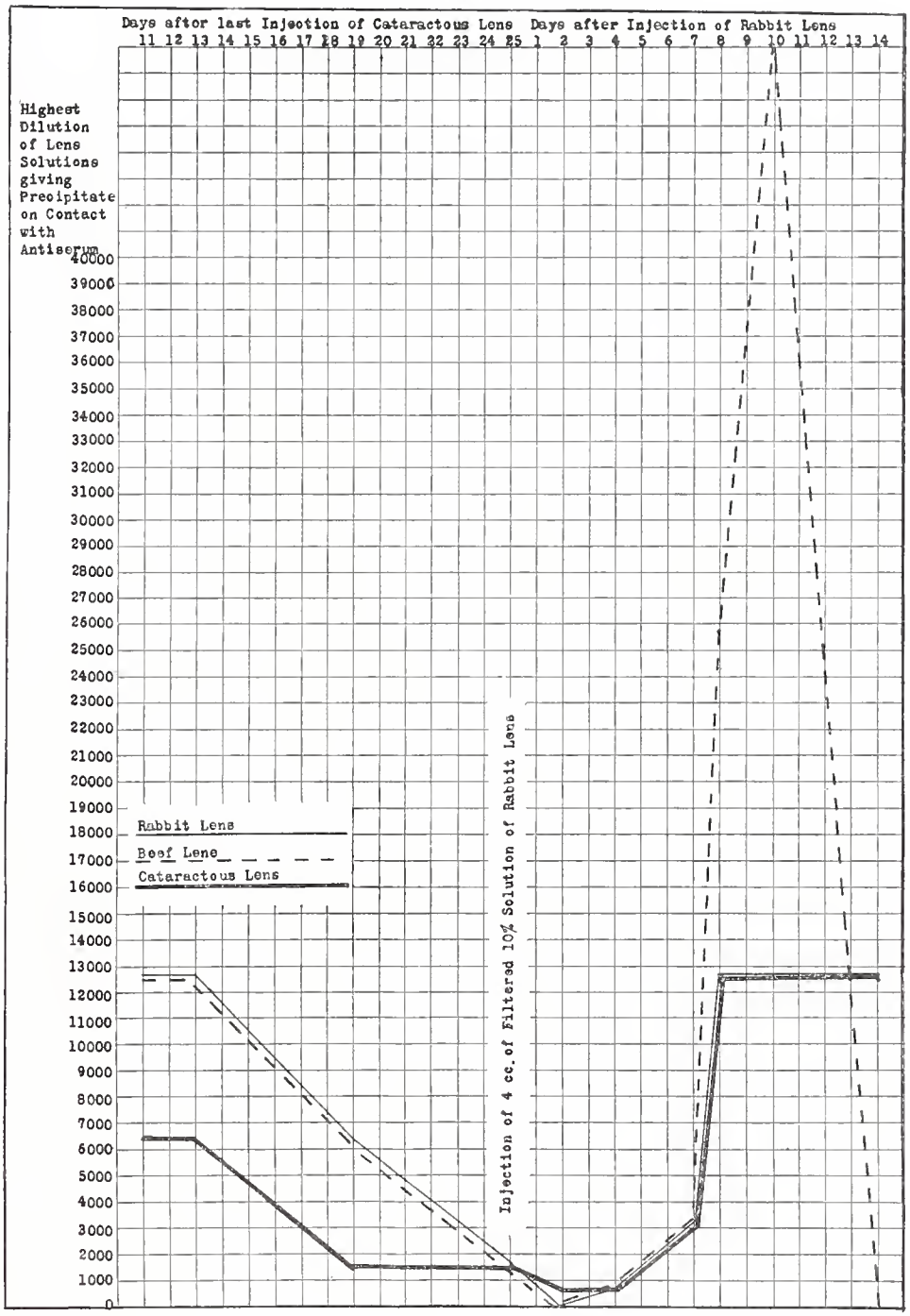

Chart 1.—Production of lens precipitins following injection of rabbit lens in rabbit previously injected with human cataractous lens.

3. PRECIPITIN FROM INJECTION OF FISH LENS (MENHADEN)

Injecting rabbits with solutions of menhaden lens induces the formation of precipitins for the lens of this and other salt water fish (table 3). At the same time, but not nearly in the same concentration, precipitins for beef, human, rabbit and swine lens are also called forth. Serum against beef, human, or swine lens also contains small amounts of precipitin for menhaden lens. This result is in harmony with earlier observations by Uhlenhuth and others.[4] On mixing precipitin serum for menhaden lens with equal parts of approximately 1 : 200 solution of this lens and letting the mixture stand for some hours, a heavy precipitate forms which carries down all the precipitins in the antiserum. Mixing the antiserum in the same way with solutions of beef, human,

TABLE 4

PRECIPITINS IN SERUM OF RABBITS INJECTED WITH LENS OF MENHADEN

Antigens	Original Serum	Serum After Treatment with Menhaden Lens	Serum After Treatment with Beef, Human, Rabbit or Swine Lens
Menhaden lens	24,000	200	6,400
Beef lens	2,400	0	0
Human lens, normal	3,200	0	0
Human lens, cataractous	1,600	0	0
Rabbit lens	6,400	0	0
Swine lens	3,200	0	0

swine, or rabbit lens removes in each case the precipitins for the mammalian lenses mentioned, leaving the precipitin for the fish lens in considerable strength (table 4). This result indicates that while the fish lens contains antigenic elements of the same kind as the mammalian lens, it also contains antigenic elements that are different and specific for fish lens. As the serum against menhaden reacts with beef and swine crystallins, and anticrystallin serum may react with menhaden lens, it seems that these crystallins constitute at least a part of the element common to both fish and mammalian lens. Obviously the precipitin reaction may be used to study the lens content in different species of alpha and beta crystallins as well as of other antigenic substances.

SUMMARY

The constituents of the lens called alpha and beta crystallins may be obtained in such form that they are precipitinogenically wholly distinct

[4] Kolle u. Wassermann Handbuch d. path. Mikroorganismen, 1914, 3, p. 257.

from each other, but each seems to be practically identical in the different mammalian lenses examined by us. Their presence in the lens of various species, including certain fishes, explains, at least in part, the organ-specificness of lens precipitins. In other words, the lens of different species always contains the same two chemically and antigenically distinct proteins.

When previously under the antigenic influence of lens of other species, rabbits may produce lens precipitins on injection of rabbit lens. Normal rabbits may react with precipitin formation to injection of rabbit crystallins.

As illustrated by the menhaden, fish lens may have antigenic elements in common with the mammalian lens, but it also contains larger amounts of other antigenic constituents that seem to be of a more limited specificness.

FURTHER OBSERVATIONS ON PRECIPITIN REACTION OF BENCE-JONES PROTEIN

Ludvig Hektoen and William H. Welker

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The studies of the immune reactions of Bence-Jones protein have not given concordant results. The earlier observers (Abderhalden and Rostoski,[1] Boggs and Guthrie [2]) by injecting Bence-Jones proteins in rabbits obtained precipitins that reacted not only with this protein but also with human serum proteins. Massini [3] noted a quantitative difference between Bence-Jones protein and blood serum proteins by means of complement fixation. He suggests that Bence-Jones is closely related yet different from the serum proteins, and that the fixation test is not suited for differentiation except with pure preparations. Micheli [4] studied a Bence-Jones protein in the urine, blood, and serous fluids of a patient with lymphosarcoma of the large intestine. Anaphylactic experiments and fixation tests with what he calls strictly pure Bence-Jones protein, as determined by careful chemical tests, and with human serum gave inter-reactions which Micheli explains as due to the presence in the Bence-Jones protein of two principal receptors, one with the same antigenic properties as the proteins in normal serum; the second, more abundant, being the specific Bence-Jones antigen.

In the serum of rabbits injected with urine from cases of myeloma, Hektoen [5] demonstrated specific precipitin reacting with urine or serum containing Bence-Jones protein, but not with normal human serum. Bayne-Jones and Wilson [6] found that while noncrystallin forms of Bence-Jones protein, isolated from urine by salting-out or other precipitation methods, contained traces of serum proteins, spontaneously crystallizing Bence-Jones protein, betrayed a specific protein, and was not acted on by antiserum to human serum.

The claim by Hektoen [5] that Bence-Jones protein is a distinct antigen is based on the results of absorption experiments as illustrated in table 1.

Received for publication, Dec. 11, 1923.

[1] Ztschr. f. physiol. chemie, 1905, 46, p. 125.
[2] Am. Jour. Med. Sc., 1912, 144, p. 803.
[3] Deutsch. Arch. f. klin. Med., 1911, 104, p. 29.
[4] Haematologica, 1921, 2, p. 1.
[5] Jour. Am. Med. Assn., 1921, 76, p. 929.
[6] Bull. Johns Hopkins Hosp., 1922, 33, p. 37; Wilson: J. Biol. Chem., 1923, 56, p. 203.

By mixing urine or serum containing Bence-Jones protein with the serum of a rabbit injected with human serum, and throwing down the precipitate, the proteins reacting with that antiserum were removed while the Bence-Jones protein was left behind in reduced amount. By adding to the antiserum obtained from a rabbit injected with myeloma urine, which contained also some ordinary protein, an equal quantity of human serum, diluted 1 to 200 with salt solution, and throwing down the precipitate that formed, the precipitins for human serum were removed while a fair amount of Bence-Jones precipitin was left behind. The difficulty mentioned by Bayne-Jones and Wilson [6] of too great

TABLE 1

DEMONSTRATION OF SPECIFIC BENCE-JONES PRECIPITIN BY SPECIFIC ABSORPTION

Antigens		Precipitin Serum of Rabbit Injected at Intervals of 3 to 4 Days Intravenously with 4, 8, 12, 16 C c. of Urine of Myeloma Patient		Precipitin Serum of Rabbit Injected with Human Serum
		As Separated	After Adding an Equal Part of Human Serum 1:320 and Removing Precipitate	
Urine of myeloma patient	As passed	16,000	1,280	128
	After treating with serum of rabbit injected with human serum—urine, salt solution, antiserum aa—and removing precipitate	4,000	1,280	0
Serum of myeloma patient	As separated	20,000	1.280	16,000
	After treating with serum of rabbit injected with human serum and removing precipitate	2,400	300	0
Human serum		8,000	0	16,000

The figures in all tables give the highest dilutions in salt solution of the antigens in which definite precipitates formed after one hour at room temperature.

loss of precipitin from dilution of the antiserum is avoided by this method. The further danger of overlooking specific reactivity because of absence of reactions in low dilutions of the antigen, is always present if tests are not carried out in an adequate series of progressive dilutions.

In experiments to obtain a pure Bence-Jones protein, we used urine from the case of myeloma and Bence-Jones proteinuria under the care of Dr. C. A. Parker in the Cook County Hospital.[5] Toluol was added as a preservative. Urine was brought to the laboratory daily where it was raised to a 40% saturation with saturated solution of ammonium sulphate. After standing 24 hours the slight precipitate which invariably formed was filtered off and the filtrate raised to a 60% saturation.

The precipitate which formed between these two saturation limits is the material on which this work was carried out.

In order to study whether it concerned a mixture of different proteins having different characteristics, the fractional precipitation method originally devised by Marshall [7] was used. The method consists of adding saturated ammonium sulphate solution drop by drop while the liquid is being thoroughly mixed by mechanical means. This prevents any excessive concentration of ammonium sulphate at any given point in the solution and results in a precipitation more nearly approximating the limits than the old method of rapidly adding the ammonium sulphate solution with more or less effectual stirring. We attempted to separate the 40-60% precipitate into the 40-45, 45-50, 50-55, and 55-60 fractions. In further application of this fractional method we tried to continue

TABLE 2

TEST OF PRECIPITIN SERUM FOR BENCE-JONES PRECIPITATE

Antigens	Precipitin Serum of Rabbit Injected with 5% Solution of Bence-Jones Protein Precipitate		
	As Separated	After Adding an Equal Part of Human Serum 1:320 and Removing Precipitate	After Adding an Equal Part of Solution of Bence-Jones Protein Precipitate 1:640 and Removing Precipitate
Urine of myeloma patient	25,000	800	0
Serum of myeloma patient	16,000	80	0
Bence-Jones protein	100,000	80,000	0
Human serum	16,000	0	0
Human pseudoglobulin	100,000	40,000	0

the precipitation of each 5% fraction until in a solution of the particular fraction no further precipitation occurred outside of its own limits. In this attempt we encountered marked difficulty, because the fractions apparently shifted in their precipitation limits owing partly perhaps to slight changes in the temperature at which the experiments were carried out and also possibly to a change in the dispersion of the colloids in solution. The net results in the attempts to obtain 5% fractions gave only small quantities of the final products.

Rabbits injected intravenously with solutions of precipitated Bence-Jones protein gave serums most of which reacted as illustrated in table 2. The admixture of pseudoglobulin there evident persisted in a large series of experiments with different Bence-Jones fractions and antiserums. Pseudoglobulin reacted with serum against solutions of

[7] Science, N. S., 1904, 19, p. 715.

Bence-Jones precipitates, and vice-versa. Tests with human serum albumin were made at the same time, and nearly always with negative results. As expected, Bence-Jones antiserum reacting with pseudoglobulin reacted with human serum, and also on some occasions with euglobin. In some cases, serum against Bence-Jones precipitate, when mixed with equal parts of 1 to 200 dilution of human serum, the resulting precipitate being removed by centrifugation, lost its precipitin

TABLE 3

REMOVAL OF PRECIPITINS FOR PSEUDOGLOBULIN, ETC., IN ANTISERUM FOR BENCE-JONES PRECIPITATE BY ABSORPTION WITH HUMAN SERUM

Antigens	Serum of Rabbit Injected with Bence-Jones Protein	
	Original	After Mixing with Equal Quantity of Human Serum 1:200 and Removing Precipitate
Bence-Jones protein	32,000+	10,000+
Euglobulin	8,000	0
Pseudoglobulin	32,000+	0
Serum	32,000+	0

TABLE 4

PRECIPITIN SERUM FOR CRYSTALLIZED BENCE-JONES PROTEIN

Antigens	Serum of Rabbits Injected with 1.5, 2, 3, 4, and 6 C c. of 1% Solution of Crystallized Bence-Jones Protein, Intravenously, at Three Day Intervals		
	Serum A	Serum B	
		As Obtained	After Absorption with Albumin or Pseudoglobulin, 1:1,000
Bence-Jones protein	1,000,000	1,000,000	500,000
Englobulin	0	0	0
Pseudoglobulin	0	1,000	0
Albumin	0	100,000	0
Normal serum	0	1,000	0
Myeloma urine	2,500 (2 years old)		

for human serum, euglobin and pseudoglobulin, but retained a high precipitin strength for Bence-Jones protein (table 3).

It is evident that the precipitates we used as antigens did not contain Bence-Jones protein exclusively, and our results naturally suggest the inference that Abderhalden and Rostoski,[1] Boggs and Guthrie,[2] Massini,[3] and Micheli[4] also used preparations that contained other antigens besides Bence-Jones protein. In fact, other observers seem not to have tested immunologically the purity of their Bence-Jones antigens.

During the fractionations of the Bence-Jones urine globular masses were observed on the walls of one of the vessels, which on microscopic examination had the characteristic appearance of egg albumin at the beginning of crystallization before spicules begin to form. Attempts were now made to crystallize the Bence-Jones protein by the Hofmeister method for preparing crystallized egg albumin. Solution of Bence-Jones protein in water was mixed with a saturated solution of ammonium sulphate until a slight permanent opalescence appeared;

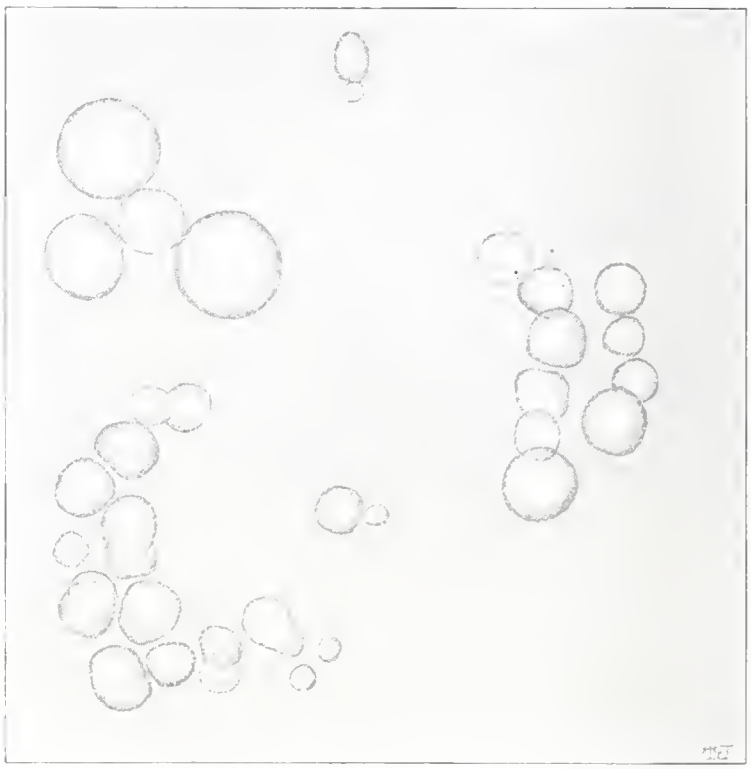

Fig. 1.—Bence-Jones crystals, early stages.

the solution was then filtered and the mixture subjected to slow spontaneous evaporation. Figures 1, 2 and 3 illustrate the crystals that appeared at successive stages of the evaporation. Our final precipitin experiments were made with solutions of the crystallized Bence-Jones protein. As a rule, these solutions did not react with antiserums to human serum, albumin, euglobulin or pseudoglobulin; in some instances, low dilutions reacted with anti-albumin and antipseudoglobulin serums. When first drawn, serum of rabbits injected with freshly made solu-

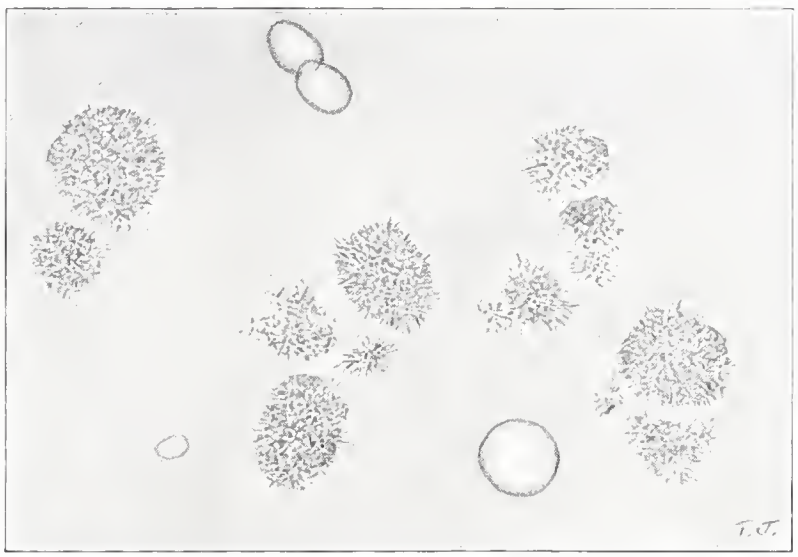

Fig. 2.—Bence-Jones crystals.

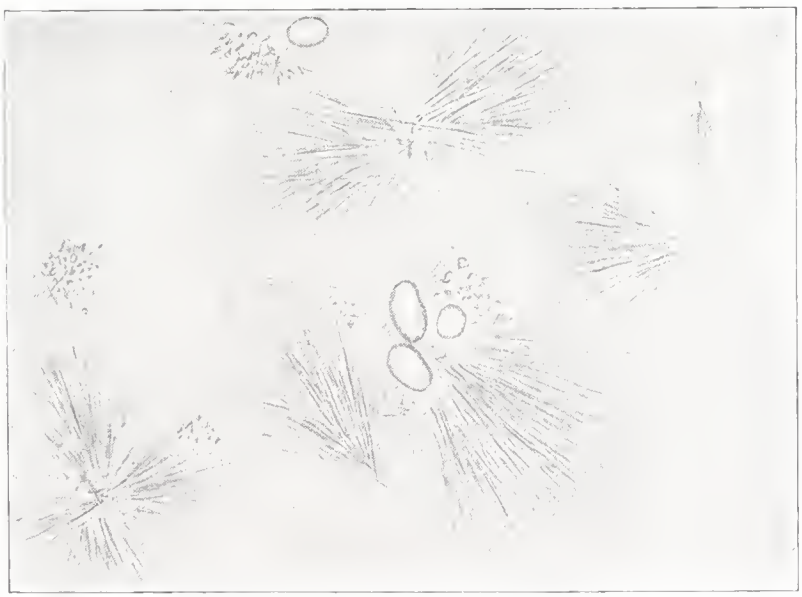

Fig. 3.—Bence-Jones crystals.

tions of Bence-Jones crystals (table 4) at times contained precipitins for albumin and pseudoglobulin in small amounts, but these precipitins were clearly of a minor nature, being as shown in the table not present in all antiserums and easily removed by specific absorption.

According to Krauss,[8] Bence-Jones protein has been observed in the crystallin form 7 times. In 3 of the 7 cases the crystals formed spontaneously, but one of these three is now known not to belong in the Bence-Jones group.[9] The Bence-Jones protein used in our experiments gives chemical tests as follows in 1% solution of the purified but not crystallized product: strong biuret, Millon, and xanthoproteic reactions; moderate Hopkins-Cole, negative Liebermann, and faint Molisch reactions; concentrated hydrochloric acid and sucrose with heat produce a brownish coloration; potassium hydroxid and lead acetate with heat gave a faint trace of loosely bound sulphur. On heating a 1% solution, cloudiness appears at 50 C., which does not clear up wholly at the boiling point, a faint opalescence remaining even after adding sodium chloride. The precipitate reappears on cooling.

It is evident that our Bence-Jones protein differs from that studied so carefully by Krauss in containing much less of the carbohydrate group and also less loosely bound sulphur. These chemical differences are of special interest in view of the differences in immune reactions of Bence-Jones proteins noted by Bayne-Jones and Wilson.[6]

SUMMARY

Precipitin tests of urine and serum in myeloma, aided by specific absorption methods, indicate that Bence-Jones protein is distinct and different from normal blood proteins. On precipitation with ammonium sulphate, the Bence-Jones protein in the urine of a myeloma patient was not obtained in pure form, being mixed especially with pseudoglobulin, but after crystallization by Hofmeister's method for preparing crystallized egg albumin, traces only of pseudoglobulin and albumin were retained in solutions of the crystals, and on injection of rabbits these solutions called forth specific Bence-Jones precipitins in large quantities. The difference in chemical behavior of the Bence-Jones protein under discussion and of that studied by Krauss [7] suggests that immunologic varieties of this protein depend on recognizable chemical differences.

[8] Deutsch. Arch. f. klin. Med., 1921, 137, p. 257.

[9] Evertt, H. S., Bayne-Jones, S., and Wilson, D. Wright: Precipitin Reactions of a Crystalline Globulin from Human Urine, Bull. Johns Hosp., 1923, 34, p. 385.

IMMUNOLOGIC SIGNIFICANCE OF VITAMINS

IV. INFLUENCE OF LACK OF VITAMIN C ON RESISTANCE OF THE GUINEA-PIG TO BACTERIAL INFECTION, ON PRODUCTION OF SPECIFIC AGGLUTININS AND ON OPSONIC ACTIVITY

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The significance of the diet in maintaining a normal resistance to infection has lately assumed increasing importance. In both human and veterinary pathology a lowered resistance has been shown to result when certain defective dietaries are employed. The vitamins, A and B particularly, have received considerable attention, although the significance of vitamin C has up to the present time remained more or less obscure.

This situation has probably resulted from the difficulty in securing satisfactory experimental conditions. Such an investigation is at present restricted to man, guinea-pig, or monkey. This practically limits any investigation to the guinea-pig, an animal relatively susceptible to bacterial infection, and therefore an animal in which variations in resistance are difficult to determine. Moreover, difficulty arises in determining whether death in a scorbutic guinea-pig has resulted from infection or vitamin deficiency.

Findlay [1] recently has dealt with the significance of vitamin C and has found scorbutic guinea-pigs somewhat less resistant to bacterial infection with B. coli, Strep. hemolyticus, Staph. aureus and the pneumococcus, and has ascribed this to the "degenerative changes and feeble leucoblastic reaction seen in the bone marrow of chronic scurvy guinea-pigs."

EXPERIMENTS

Scorbutic guinea-pigs received a ration of ground oats and wheat bran ad libitum and daily approximately 50 c c. of autoclaved whole milk. Loss of weight followed by acute scurvy usually resulted in from 4 to 6 weeks. A small amount of lemon juice was occasionally administered to an animal in distress in order to bring the animals to the experimental stage in a uniform condition, as nearly as is possible,

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[1] Jour. Path. & Bacteriol., 1923, 26, p. 1.

judging from macroscopic or clinical appearances. Control guinea-pigs received a ration of alfalfa hay, cabbage leaves, oats and carrots and in addition unautoclaved milk ad libitum. The animals were allowed the dietary for from 5 to 6 weeks before they were injected with a suspension of the particular organism under investigation. As avitaminic guinea-pigs lose weight rapidly, it was impossible to obtain scorbutic and normal pigs of the same weight and age. For this reason the ages of the animals were largely ignored and the weights chosen approximately equal. This did not in any way invalidate the results of the experiment; in fact, it afforded more definite conclusions for the reason that if the age of an animal is a factor involved in its resistance, it is undoubtedly in favor of the scorbutic animals. The younger control pigs would be more susceptible than the older scorbutic animals, disregarding, of course, their condition of vitamin deficiency. The scorbutic pigs were, therefore, as a rule somewhat older than the controls.

The pneumococcus injected was isolated from the lungs of a moribund rat. This organism was gram-positive, produced methemoglobin on blood agar, but no definite zone of hemolysis, and was not agglutinated by pneumococcus serums, types 1, 2 or 3. It was highly fatal to white mice, and virulence was maintained by preserving the livers of mice which had died from the infection. Guinea-pigs were injected intraperitoneally with a supernatant suspension made by macerating a freshly infected mouse liver in salt solution.

The strain of anthrax bacillus used for injections was chosen from several of various degrees of virulence. This particular strain was of low virulence and regularly killed 250 gm. guinea-pigs when injected intraperitoneally in 0.14 c c. doses of an 18-hour broth culture.

Experiments with Pneumococci.—Preliminary experiments with several series of healthy pigs, injected intraperitoneally with various doses of pneumococci indicated that the minimal lethal dose lay near 0.18 c c. for pigs weighing from 250-300 gm. A supernatant suspension of pneumococci from a freshly infected mouse liver and peritoneal fluid was employed.

Several preliminary experiments were then made to determine the dose of pneumococci which on intraperitoneal injection into 250-300 gm. guinea-pigs would produce death in the scorbutic animals but not in the controls. After several experiments of this nature, the results shown in table 1 were obtained and indicate the amount of pneumococcus suspension which gave the most differential results. Smaller

doses apparently were tolerated as well by scorbutic pigs as by healthy controls, while larger injections proved fatal to both. There are no striking differences noted, and any break in the resistance in scorbutic pigs is not a collapse in any way. However, no such collapse in the resistance of an animal to infection is to be expected. The break would probably be gradual and progressively increase with conditions. Of

TABLE 1

RESISTANCE OF THE GUINEA-PIG LACKING VITAMIN C TO PNEUMOCOCCI

Pig	Weight, Gm.	Condition	Rectal Temperature Fahrenheit	Pneumococcus Injections Ml.	Results	Remarks
1	200	Scorbutic	102.6	0.18	Lived	Average days for deaths, 3
2	200	Scorbutic	98.6	0.18	Died 2d day; septicemia	
3	270	Scorbutic	99.1	0.18	Died 3d day; septicemia	
4	250	Scorbutic	100.5	0.18	Died 2d day; septicemia	
5	250	Scorbutic	100.6	0.18	Died 4th day; septicemia	
6	245	Scorbutic	100.9	0.18	Died 5th day; peritonitis	
7	265	Scorbutic	103.4	0.18	Lived	
8	275	Scorbutic	102.9	0.18	Died 3d day; septicemia	
9	280	Scorbutic	99.3	0.18		
10	275	Control	102.6	0.18	Lived	Average days for deaths, 4.6
11	270	Control	103.1	0.18	Died 2d day; septicemia	
12	265	Control	102.6	0.18	Died 5th day; peritonitis	
13	245	Control	103.4	0.18	Died 4th day; septicemia	
14	270	Control	103.2	0.18	Lived	
15	270	Control	102.9	0.18	Died 6th day; septicemia	
16	255	Control	102.8	0.18	Died 6th day; septicemia	
17	250	Control	103.4	0.18	Lived	

TABLE 2

BLOOD IN SCORBUTIC AND HEALTHY GUINEA-PIGS

No. of Pigs Averaged	Red Corpuscles	White Cells	Polymorphonuclears, Percentage	Eosinophils, Percentage	Basophils, Percentage	Mononuclears, Percentage	Lymphocytes, Percentage	Arneth Index
14 scorbutic.....	4,890,000	8,450	52.3	1.1	0.2	2.4	44.0	52.5
5 controls.....	5,030,000	8,740	44.7	0.9	0.3	2.4	52.7	56.5

the animals shown in table 1, three control and two scorbutic animals survived, while of the fatal cases, the controls managed to survive 4.6 days on an average and scorbutic pigs 3 days. The scorbutic pigs in this experiment received 1 c c. of lemon juice each daily for 3 days prior to the giving of injections. The animals had been on the dietary 4 weeks and 4 days at the time of the injections.

The blood in 14 scorbutic and 5 healthy pigs showed no significant differences. The results are averaged in table 2. The greater number

of lymphocytes in the blood of the controls is probably due to age differences. Occasionally there occurs in scorbutic animals during the development of disease an increase in the red blood cells, probably a relative condition of no specific significance in scurvy since it occurs in rats lacking vitamins A or B. Eliminating these occasional increases, the average number of erythrocytes is somewhat less than the average found in healthy animals. The leukocytes also show a slight reduction in scorbutic animals. The presence of myelocytes in the blood stream is rarely noted.

The Arneth index was determined by counting the nuclear lobes of 200 leukocytes (disregarding lymphocytes). The index was taken

TABLE 3

Resistance of the Scorbutic Guinea-Pig to Bacterium Anthracis

Pigs	Weight, Gm.	Condition	Rectal Temperature, Fahrenheit	Results	Remarks
24	230	Scorbutic	98.3	Died in 36 hours	Average days for deaths, 2.6
25	250	Scorbutic	99.7	Died in 36 hours	
27	240	Scorbutic	100.1	Died in 48 hours	
28	235	Scorbutic	100.1	Died in 48 hours	
33	255	Scorbutic	100.3	Died in 72 hours	
34	240	Scorbutic	101.3	Died in 96 hours	
36	260	Scorbutic	101.9	Died in 96 hours	
41	240	Control	102.7	Died in 48 hours	Average days for deaths, 3
42	260	Control	103.1	Lived	
43	245	Control	103.4	Died in 48 hours	
44	235	Control	102.9	Lived	
46	255	Control	103.3	Died in 72 hours	
51	245	Control	102.4	Died in 96 hours	
52	265	Control	102.6	Died in 96 hours	

as the sum of the percentages of leucocytes with 1 and 2 lobes and one-half of those with 3 lobes. The index determined in 14 scorbutic and 5 normal healthy guinea-pigs was slightly lower in the case of scorbutic animals. Findlay [1] noted the presence of areas of marked degenerative changes in the bone marrow and feeble leukoblastic response.

Experiments with Anthrax.—The experiments employing B. anthracis yielded results similar to those with the pneumococcus. Table 3 summarizes one of the final experiments with B. anthracis of low virulence; 0.1 c c. of an 18-hour broth culture was injected intraperitoneally. The scorbutic animals failed to show the resistance manifested by the healthy controls. The break in resistance of the scorbutic animals is not striking; it is, however, definite and deter-

minable. These results offer confirmation to the numerous publications of clinical interest indicating a close relationship of mortality to deficient dietaries.

In table 4 are summarized the results of 4 final series of experiments using the pneumococcus or B. anthracis. Two of these represented experiments with the pneumococcus and two with B. anthracis. The inoculations were not identical or of the same quantity in all 4 experiments. However, for every scorbutic animal receiving certain treatment, a control received a similar injection; thus the results are comparable. As pointed out in the case of the break in resistance of animals suffering from the lack of vitamins A and B, the reduced body temperature resulting in guinea-pigs on a vitamin C deficient ration is undoubtedly of primary significance in accounting for the reduced

TABLE 4

SUMMARIZED RESULTS OF INJECTIONS OF PNEUMOCOCCUS AND B. ANTHRACIS

Treatment	Died		Number Dying after Days							Average Days Dying	Lived	
	No.	%	1	2	3	4	5	6	7		No.	%
Scorbutic (34).....	29	85	5	12	7	4	1	0	0	2.4	5	15
Controls (43).....	32	74	2	8	6	7	3	5	1	3.6	9	26

resistance. We have found the degree of resistance to be closely correlated with the body temperature. This results indirectly through the effect of temperature reduction on phagocytosis, bacterial activity of blood serum and body fluids, and in the case of animals with a normal body temperature above 37 C., a more favorable environment for the development of ordinary pathogenic bacteria.

Production of Agglutinins in the Scorbutic Guinea-Pig.—The influence of vitamins on the production of antibodies has been studied by Guerrini,[2] Zilva,[3] and Werkman.[4] Guerrini came to the conclusion that pigeons fed on a diet of polished rice lose their ability to elaborate agglutinins. Zilva found no disturbance in the production of agglutinins or hemolysins in the rat fed on a diet free from vitamin A or B; or in the guinea-pig on a ration low in vitamin C. Werkman concluded that rats and rabbits lacking vitamin A or B elaborated agglutinins, precipitins, hemolysins and bacteriolysins in no manner observably different from normal controls.

[2] Ann. d'iq., 1921, 31, p. 596.
[3] Biochem. Jour., 1919, 13, p. 172.
[4] Jour. Infect. Dis., 1923, 32, p. 247.

The Dreyer technic as used in a previous work [4] was employed in the agglutination tests. The guinea-pigs were given intraperitoneal injections after 4 weeks on the test rations. These injections were 0.2, 0.3, 0.4 and 0.6 c c., of a killed 18-hour broth culture of Bact. typhosum. The scorbutic animals received a small quantity of lemon juice (approximately 0.5 c c. per day) to maintain life. Tests were made on 5 scorbutic and 7 control guinea-pigs. In table 5 are given representative results obtained with 2 of the scorbutic and 2 of the control animals. No disturbance in the production of specific agglutinins for the typhoid bacillus occurs as the result of vitamin C deficiency.

TABLE 5

Production of Specific Agglutinins in Scorbutic Guinea-Pigs

Pigs	Days after Last Injection of Antigen	Titer
Scorbutic Pig 1	10	120
	6	480
	6	3,840
	8	15,360
Scorbutic Pig 2	10	60
	6	240
	6	1,920
	8	7,680
Control Pig 1	10	60
	6	240
	6	1,920
	8	7,680
Control Pig 2	10	30
	6	120
	6	1,920
	8	7,680

Phagocytic Activity in the Scorbutic Guinea-Pig.—Infection in the avitaminic animal is often of a type normally suppressed by phagocytic activity. For this reason the phagocytic activity was determined in scorbutic and healthy guinea-pigs. Findlay and MacKenzie [5] and Werkman [6] recently concluded that normal phagocytosis in vitro is not depressed in avitaminic rats (minus A and B) although the latter suggested from the results of his investigations in vivo the importance of depressed body temperatures in determining phagocytic activity. Biondo [7] found a diminution in the opsonic index of polyneuritic pigeons. The present part of this investigation deals

[5] Biochem. Jour., 1922, 16, p. 574.
[6] Jour. Infect. Dis., 1923, 32, p. 263.
[7] Policlin. Sez. Prat., 1922, 29, p. 3.

with phagocytic activity in normal and scorbutic guinea-pigs. The experiments were in vitro using Bacteruim typhosum and Staph. albus. Leukocytes for the tests were obtained from stock animals by peptone injection and pooled. Test serums were obtained from 4 scorbutic and from 4 healthy animals and pooled into their respective groups.

The method of Wright was employed in the opsonic determinations. The results reveal no significant differences in the phagocytic activities of normal and scorbutic guniea-pigs determined in vitro. There exists, therefore, no rupture in the phagocytic mechanism. Depression is the result of unfavorable environmental conditions, reduced body temperature playing an important part in such conditions. The phagocytic indexes were: for Bacterium typhosum, 0.87; for Staph. albus, 1.31 (scorbutic pigs); control serum for Bacterium typhosum, 1.05; for Staph. albus, 1.26.

The determination of immune opsonins offers no evidence of subnormal values in scorbutic animals. Three pigs that had received the scurvy producing ration for 3 weeks were given intraperitoneal injections of 0.2, 0.3, 0.4 and 0.5 c c. of a killed 18-hour broth culture of Bacterium typhosum at 4 day intervals. Three controls received the same treatment. Phagocytic indexes were determined 1 week after the last injection. One milliliter of lemon juice was given to each scorbutic animal daily from the time of the first injection until the last. The phagocytic indexes of the pooled serums were: scorbutic, 3.61; control, 3.46. No injury to the opsonic mechanism, therefore, results from the lack of vitamin C.

SUMMARY

Guinea-pigs suffering from the lack of vitamin C experience a definite and determinable though not marked break in their resistance to infection by the pneumococcus and B. anthracis. The reduced body temperature is of primary significance in accounting for the reduced resistance to infection.

Guinea-pigs lacking vitamin C reveal no differences in their ability to produce specific agglutinins for the typhoid bacillus from that of healthy animals.

Investigation of the phagocytic activities in guinea-pigs lacking vitamin C revealed no injury to the phagocytic mechanism as the result of vitamin C deprivation.

PNEUMONIA DEATH RATE AND TYPES OF PNEUMOCOCCI IN NORWAY

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Avery, Chickering, Cole, and Dochez [1] give the death rate of patients with pneumonia not receiving serum treatment in the hospital of the Rockefeller Institute as nearly 30%; the death rate of type 1 pneumonia they give as 25%, of type 2 as 32%, of type 3 as 45% and of type 4 as 16%. These are hospital figures and consequently probably somewhat higher than the figures for cases outside hospitals. The death rate from pneumonia in Norway is undoubtedly lower than in the United States, and we have made observations on the types of pneumococci in Norway in order to ascertain whether there is any difference in the distribution of the different types in the two countries. We obtained sputum from the hospitals in Christiania and from private practitioners, which we studied according to the accepted technic. Immediately on arrival in the laboratory the sputum is examined as to the various forms of bacteria present; a small particle is then emulsified in broth and injected into the peritoneal cavity of a white mouse; at the same time, a loop of sputum is streaked on the surface of a plate of agar with rabbit blood 7%. As soon as dead, and usually death occurs within 20 hours, the peritoneal cavity of the mouse is washed out with salt solution and the suspension centrifugated so as to throw down the cells that may be present, and an agglutination test made with serum obtained either from the Rockefeller Institute or from the Health Department of New York City. The serum is used in a dilution of 1 : 10, and the results are read immediately or after one hour in a water bath at 37 C. Further identification of the pneumococcus is made according to its growth on the surface of the blood agar, the fermentation of inulin and its solution in bile. According to this technic, 100 cases of typical lobar pneumonia gave 44 cases of type 1, 33 of type 2, 6 of type 3, and 17 of type 4. There is a close correspondence in these results with the results obtained in the United States, type 1 being apparently more prevalent in Norway, but on account of the comparatively small number of cases examined by

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[1] Acute Lobar Pneumonia, Monograph 7, Rockefeller Institute for Medical Research, 19.

us, which include two small epidemics of pneumonia in military barracks due to type 1, this difference may be largely accidental. It must not be overlooked that the different types of pneumococci may vary in the same place from time to time.[1] According to Christensen,[2] the type incidence of pneumococci in Denmark runs as follows: type 1, 33%; type 2, 27%; type 3, 8%; type 4, 32%; type 1 being the dominating type, but less markedly so than in our series. The cultural characteristics of the pneumococci we studied seem to be the same as those observed by American workers. The fermentation of inulin was subject to similar irregularities in our hands, as described by Avery and his coworkers [1] and by Berry.[3]

In order to obtain further information in regard to the death rate of pneumonia in Norway, we made special efforts to learn of the fate of all the patients from whom sputum was examined. The results are given in table 1. All the cases in table 1 were cases of typical acute lobar pneumonia, the pneumococcus in each case being identified and typed as described.

TABLE 1

ANALYSIS ACCORDING TO PNEUMOCOCCUS TYPE AND DEATH RATE OF 100 CASES OF PNEUMONIA

Type	Death Rate in Percentage of Type	Empyema in Percentage of Type	Abscess of Lung in Percentage of Type	Otitis in Percentage of Type
1	9	11.3	0	2.3
2	6	6	3	0
3	16.6	0	0	0
4	6.6	6	0	0

Table 1 shows that pneumococcus type 3 causes the most dangerous form of pneumonia in Norway just as in the United States. It is, however, the pneumococcus type that most rarely causes pneumonia, type 1 being the most important type because it causes more pneumonia than any of the other types and more complications. Considering the deaths in our series, it is noteworthy that only 8 deaths occurred in 100 cases, including one death from abscess of the lung following pneumonia. This is a much lower death rate than in the United States in patients who have not received serum treatment. In Christensen's [2] series from Denmark the case death rate was 12%.

To study further the death rate of pneumonia in Norway, we have gone over the statistics of morbidity and mortality of pneumonia in

[2] Communications de l'Institut Serotherapique de l'Etat Danois, 1923, 14.
[3] Jour. Infect. Dis., 1907, 4, p. 93.

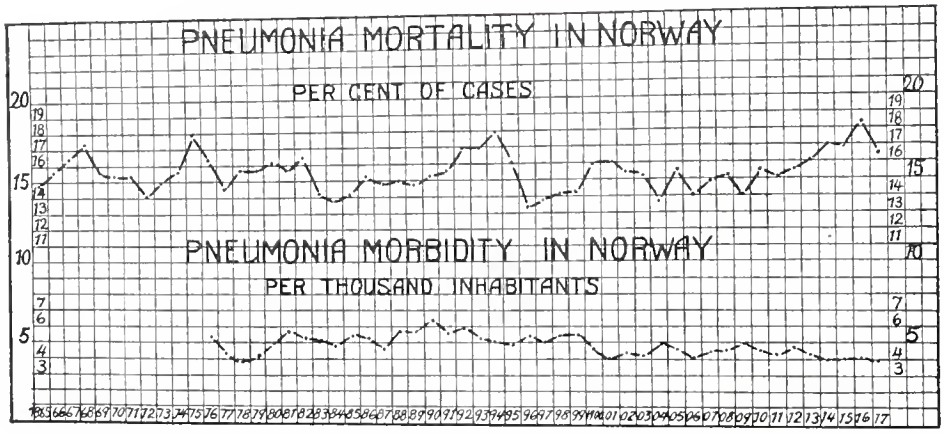

Chart 1.—Pneumonia mortality and morbidity in Norway.

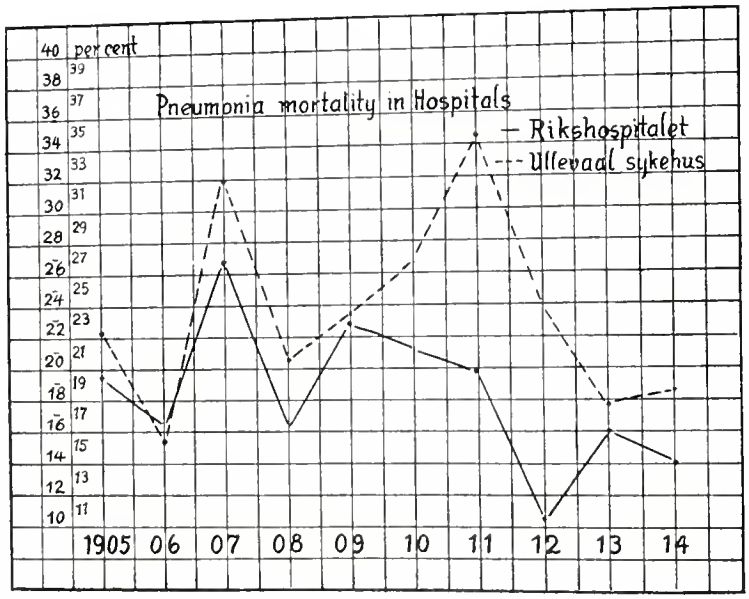

Chart 2.—Percentage of pneumonia mortality in two hospitals in Christiania.

Norway from 1865 to 1917, the year before the influenza pandemic. As shown in chart 1, the morbidity averages about 5 per 1,000 inhabitants, and the case death rate runs from 13 to 18%. This chart is based on the figures sent in by physicians all over the country, and such figures tend to indicate a higher death rate than is actually the case because patients with severe cases nearly always receive medical care, and consequently are listed as cases of pneumonia. Patients with milder cases, especially formerly, are more likely to be left to take care of themselves, and consequently are not reported. Our figures include also patients with pneumonia treated in hospitals, and as shown in chart 2 the death rate in the hospital cases is somewhat higher, averaging about 20%, and subject to greater fluctuation than the death rate of patients from the country as a whole.

Why the death rate in our series of 100 cases is only 8%, we cannot say. We can say, however, that all were cases of real pneumococcus pneumonia, and that we excluded 2 fatal cases of streptococcus pneumonia that ordinarily would have been classed as lobar pneumonia and thus would have served to raise the death rate in the statistics. We repeat that Christensen [2] in Denmark found the death rate to be 12% and that he emphasizes that in Denmark penumonia is milder than in the United States. It is of interest that there is this difference in the death rate of pneumonia in Norway and Denmark as compared with the United States, and especially since the same type distribution of pneumococci seems to exist. With its wet and cold climate Norway naturally seems more likely to a heavy death rate from pneumonia.

The question arises whether the pneumococci in the United States are more virulent than those in Norway. We have made virulence tests of 24-hour old broth cultures of freshly isolated strains of type 1 and type 4; in the case of type 1, 0.000001 c c. killed in 5 days or so and a strain of type 4 killed in 48 and 72 hours in doses of 0.001 c c. It consequently does not seem probable that the virulence of the pneumococci is less in Norway than in the United States, and the reason for the smaller death rate in Norway must be sought elsewhere. It may be that the inhabitants of Norway possess greater resistance from pneumococcus infection than the composite population in the United States, and it would be interesting to learn whether Norwegian immigrants in the United States have a smaller death rate from pneumonia than the population in American cities in general.

SUMMARY

The different types of pneumococci appear to occur in about the same relative proportion in Norway as in the United States. The death rate from typical lobar pneumonia appears to be less in Norway than in the United States.

STUDIES IN INTESTINAL BACTERIOLOGY I*

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The object of this investigation was to ascertain the variations of the bacterial flora, both aerobic and anaerobic, in respect to numbers and to species, at certain levels of the small intestine, in health and disease.

A beginning has been made to discover whether such a study may yield any significant results in the investigation of obscure diseases, especially such as have, at various times, been ascribed to focal infections in the intestines or to toxic bacterial products of alimentary tract origin.

An intensive study of one person was made, with the purpose of observing the effect of special feeding on the intestinal and also on the fecal flora, and serologic methods were employed in order to throw further light on the question of an indigenous flora, or of a possible streptococcal focus of infection.

BACTERIA IN THE INTESTINE IN HEALTH AND DISEASE

While the subject of the flora of the small intestine has been frequently under investigation, observations have usually been made on animals, on human beings directly after death, on material obtained during a laparotomy, occasionally on material derived from a fistula; or deductions have been drawn from fecal material about conditions in the upper levels in the intestinal tract. Until recently only the duodenum has been subjected to direct investigation during the processes of normal digestion.

In the first place, it is essential to determine whether there is in health a bacterial flora of the upper small intestine which is in any way distinctive either quantitatively or qualitatively; that is, whether broad limits can be set to the number of viable organisms at any level, and whether these organisms belong to certain species, while representatives of other species, perhaps normally present at different levels, are here absent, and whether there is any technic by which we can satisfactorily investigate these points. When they have been determined the relationship of an altered bacterial picture to pathologic conditions may assume

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diagnostic significance. For instance, intestinal focal infections by various bacteria, especially the nonhemolytic streptococci, have figured largely in recent literature as associated with a wide range of disease conditions, and sometimes as directly causing them.

According to Norman and Eggston,[1] Cardio-vascular renal conditions, arterial diseases, essential high blood pressure, low blood pressures, the rheumatisms, some forms of eczema, diabetes, etc., are associated with infections of the digestive tract. Mutch[2] found chiefly streptococci, often abnormal forms of colon bacilli, and in a few cases staphylococci, as centers of intestinal infection in 200 cases of chronic arthritis. Cotton and Draper[3] believe that they have linked the functional psychoses with streptococcal infections, chiefly of the stomach and colon. These writers assert that removal, or when that is impossible radical treatment of the intestinal focus of infection, will also relieve the symptoms of which these infections are the supposed cause.

Various investigators have attempted to establish the importance of one or another factor in controlling or influencing the flora of the intestinal tract. Briefly these factors are:

1. The inhibitory action of free HCl of the stomach.

2. The peristalsis of the intestine.

3. The reaction of the chyme—a resultant of the following elements: i. e., the amount of free acid passing with the stomach contents into the duodenum, the reaction of (*a*) the digestive secretions, (*b*) the food in process of digestion by the amylotic, fat-splitting, and proteolytic enzymes, and (*c*) the split products resulting from the activity of such bacteria as are present.

4. The possible bactericidal or bacteriostatic activity of the digestive secretions, or of the uninjured intestinal epithelium.

5. The antagonistic action of a possible normal flora to freshly introduced organisms.

6. The quantity and character of the food ingested.

7. The presence of focal infection in the mouth, tonsils or stomach.

Although at one period it was held that the whole intestinal tract was swarming with bacterial life, it is now generally believed that the small intestine is relatively amicrobal compared to the cecum and colon, both during and in the intervals of active digestion. Investigators, however, have differed as to the degree and causes of the low bacterial concentration.

[1] New York Med. Jour., 1922, 115, p. 449.
[2] Lancet, 1921, 2, p. 1266.
[3] Med. Rec., 1920, 97, p. 719; New York Med. Jour., 1920, 111, pp. 672, 721 and 770; Am. Jour. Psych., 1922, 2, p. 157.

The conditions existing in the small intestine may be thus somewhat schematically stated. Between the stages of gastric digestion, when the pyloric sphincter is relaxed, viable mouth and throat bacteria may be washed through the stomach into the intestine. It is generally held that during gastric digestion large numbers of, but not all, bacteria present in the food will be destroyed. Kopeloff [4] has shown that the organisms introduced with the saliva ordinarily may be a more important factor in increasing bacterial numbers in the stomach than those introduced with the food. When solid food has been eaten, it usually takes several hours for the stomach to empty completely, so that while some of the food may reach the cecum from 2½ to 3 hours after ingestion, other parts are 18 to 24 hours in passage,[5] with the result that, under ordinary dietary conditions, there will probably be a food residue in the small intestine from one meal when a second meal is ingested. Thus there will usually be a dilute substratum, apart from the digestive juices, to provide nourishment for any bacteria that may have survived.

On the nature of the food, the activity of the digestive fluids, and the rate of absorption from the intestine, will depend the value of this diluted substratum in providing nourishment for the bacteria, in favoring some, and in inhibiting others. Peristalsis, as it carries down the food, sweeps the bacteria along, although a certain number may perhaps lag behind. Since the chyme is in a fluid condition, all parts will come in contact with the intestinal epithelium, which can thus exercise any bactericidal activity that it possesses. Whereas, in the cecum, the concentration of waste products due to partial dehydration and slow peristalsis seems to favor the development of a rich flora, these conditions normally appear to be absent in the small intestine.

Bloomfield [6] has come to the conclusion that the flora normally present in the mouth proliferates in the crevices of the partially desquamating epithelium and in the orifices of the glands, and from there is expelled into the buccal secretions. He accounts for the persistence of a normal flora and the rapid elimination of large quantities of foreign bacteria artifically introduced, by the assumption of a "vital biological relationship" between the normal bacteria and the mucous membranes.

We think that it is not impossible that similar conditions may exist along the mucous membrane of the intestinal wall. Since the acid reaction of the stomach and possibly of the small intestine during car-

[4] Am. Jour. Med. Sc., 1923, 165, p. 120.
[5] Macfayden, Nencki and Siebert: Arch. f. Exper. Path. u. Pharmakol., 1890-91, 28, p. 311.
[6] Bull. Johns Hopkins Hosp., 1922, 30, p. 252.

bohydrate and fat digestion would exercise a selective action, only certain types of bacteria of those continuously introduced with the saliva or food could establish themselves on the healthy epithelium. Thus a permanent autogenous flora might gradually develop along the wall of the small intestine. During active digestion the normal flora might be masked by the chance or "wild" invaders, the conditions here being in marked contrast to those existing in the cecum and colon, while in the long run the native flora might stifle competition on the part of freshly introduced bacteria which were antagonistic in their metabolic activities. Such a condition would not exclude the possibility of a change in the picture with alterations in diet lasting over a considerable period, since different decomposition products might be unfavorable to the existing flora, or with the development of a new obligate flora in conditions associated with foci of infection or prolonged stagnation.

HISTORICAL

Normal Flora of the Small Intestine in Adults and Animals.—Gessner[7] Macfayden, Nencki and Siebert,[5] Gilbert and Dominici,[8] Marfan and Bernard,[9] Cushing and Livingood,[10] Kohlbrugge,[11] Fischer,[12] Rolly and Liebermeister,[13] Herter,[14] and Kendall[15] are among those who made observations on the normal upper gastrointestinal tract in man or in animals. In general, their results may thus be summarized: The bacterial count is low with a gradual increase from the duodenum onward. Staphylococci and some spore-bearing aerobes are common, while putrefactive organisms (anaerobes) are few, and proteins are therefore only slightly attacked by bacteria in the small intestine. Cushing and Livingood[10] believed that the flora was dependent on the bacterial features of the ingesta, and that a completely amicrobal condition could be produced.

MacNeal and Chase[16] were the first to investigate the duodenal flora of adults obtained in an aseptic manner during digestion. They used a duodenal tube with a gelatin capsule covered with shellac. Material was withdrawn one hour after the administration of an Ewald breakfast. Their patients suffered from a great variety of diseases. The count ranged from less than 100 to more than 1,000,000 viable organisms per c c. They found that gram-positive nonliquefying cocci constituted the bulk of the living flora when the colonies ranged from 40 to 1,000 per c c. Staphylococci and sarcinae were present but not in high numbers in a wide range of conditions. They found no spores, and typical B. coli in only two cases, and then only in small numbers.

Torrey,[17] after feeding a dog for 7 weeks on a balanced diet of 500 gm. of boiled rice and 100 gm. of beef-heart per day, made the following observations: The duodenum and jejunum had a low count; the chief organisms were strepto-

[7] Arch. f. Hyg., 1889, 9, p. 128.
[8] Bull. Soc. de biol., 1894, 10, I, p. 117.
[9] Presse méd., 1899, 7, p. 217.
[10] Johns Hopkins Hosp. Rept., 1900, 9, p. 543.
[11] Centralbl. f. Bakteriol., 1901, 29, p. 571; 1901, 30, p. 10.
[12] Vorlesungen über Baktieren, 1903, 2, p. 178 and p. 283.
[13] Deutsch. Arch. f. klin. Med., 1905, 83, p. 413.
[14] Bacterial Infections of Digestive Tract, 1907.
[15] Jour. Med. Res., 1911-12, 25, p. 117.
[16] Arch. Int. Med., 1913, 12, p. 178.
[17] Jour. Med. Res., 1918-19, 39, p. 415.

cocci and B. welchii. There were no evidences of putrefaction in the sediment of the sugar-free broth fermentation tube but much gas production in the glucose broth fermentation tube. In the ileum, B. coli was numerous and B. welchii very numerous, and there was much evidence of putrefaction in the sediment of the sugar-free fermentation tube.

Hoefert,[18] also using the duodenal tube, found the duodenum almost sterile in 14 patients with normal HCl and healthy bile ducts. His patients did not eat for 12 hours before swallowing the tube, and the mouth was well disinfected.

Gorke[19] examined the duodenum under aseptic conditions after from 12 to 14 hours of fasting. He found the material cultivated from half the cases sterile; among the remainder, the number of bacteria was low but the varieties were considerable. He found nonhemolytic cocci, acidophilus-like bacilli and enterococci. B. coli in normal cases was not present.

Ganter and Van der Reis[20] were the first to publish bacteriologic studies of the human small intestine below the duodenum during the normal processes of digestion. They originally used sterile metal cylinders which could be opened to take up material by means of a powerful magnet at any point in the intestinal tract, and which were then recovered from the feces. Later they used the Einhorn tube, employing a method somewhat like our own.

Van der Reis,[21] as a result of studies on 350 cases, concluded that there was an obligate flora of the small intestine. Except for isolated "wild" bacteria, he found the same general picture in all cases. In the upper intestine he observed glycophylic gram-positive lance-shaped diplococci, short and long gram-positive bacilli of the milk-souring group, and isolated gram-negative bacilli of the aerogenes group. In the middle small intestine the flora was richer, the number of glycophylic cocci gradually decreasing, and true B. coli with related gram-negative bacilli becoming more numerous. In the lower small intestine, he found the gram-negative bacteria beginning to predominate over the gram-positive.

Bogendorfer[22] has also examined the floral contents of parts of the small intestine under normal conditions of digestion, following the tube technic of Ganter. His subjects took no food between the evening meal and the withdrawal of the specimen on the following morning. He examined 15 normal cases, in which he observed the presence of an obligate flora consisting of (1) the enterococcus of Thiercelin, (2) gram-negative diplococci, (3) B. acidophilus, (4) few B. acidilactici. B. coli was normally absent until a point 250 cm. from the pylorus was reached. Often the enterococcus was the only organism in the upper tract, while 2 meters from the pylorus the flora became more complicated with B. acidilactici predominating.

N. and J. Mutch,[23] examining specimens of intestinal contents taken during laparotomies, found that normally, in the small intestine, streptococci are present in small numbers only, and that B. coli is absent from the upper levels.

Aronovitch, Coleman and Einhorn[24] have made a preliminary report on the gastric, duodenal and jejunal contents of 8 subjects. All of them were hospital cases, two suffering from arthritis. The patients had not eaten for 15 hours before the sample was taken. They found the duodenal flora of the same person more diversified than the gastric flora, and in several cases that of the

[18] Ztschr. f. klin. Med., 1921, 92, p. 221.
[19] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1922, 35, p. 279.
[20] Deutsch. Arch. f. klin. Med., 1921, 137, p. 348; München. med. Wchnschr., 1922, 69, p. 347.
[21] Klin. Wchnschr., 1922, 1, p. 950.
[22] Deutsch. Arch. f. klin. Med., 1922, 140, p. 257.
[23] New York Med. Jour., 1921, 113, p. 713.
[24] Proc. Soc. Exper. Biol. & Med., 1922, 20, p. 97.

jejunum more diversified than that of the duodenum. They observed great variation in bacterial numbers. The contents of the subject with arthritis deformans had a uniformly high count, that of the subject with chronic arthritis a much lower count. Three subjects showed a progressive increase as the tube descended, two gave evidence of no increase, or a slight one. At the lower levels the bacilli of the colon group assumed greater prominence. Streptococci were not uniformly recovered. The authors distinguish between the enterococcus and the streptococcus.

ABNORMAL AND DISEASE CONDITIONS

Effect of Gastric Hypo- and Hyper-Acidity.—Most investigators agree that the HCl of the stomach is important if not essential in keeping down bacterial numbers in the upper small intestine.

Schütz,[25] experimenting on a dog with a fistula that separated the intestine from the stomach, was convinced that the HCl of the stomach was not necessary for the prevention of putrefaction.

Tabora,[26] on the other hand, believed that the HCl of the stomach acts selectively by favoring aciduric and inhibiting putrefactive bacteria.

Kiralyfi,[27] MacNeal and Chase,[16] Hoefert[18] and Gorke[19] all found the duodenal flora in man directly influenced by the acid of the stomach, and Van der Reis[21] found fewer than the normal number of bacteria in the upper small intestine in several cases of hyperacidity and greater numbers in cases of hypo- and anacidity.

Pernicious Anemia.—Bogendorfer[22] and Gorke[19] both found large numbers of B. coli in the upper small intestine associated with this disease, while Van der Reis[21] noted proteolytic and clostridial forms in quantity in the lower small intestine.

Biliary Diseases.—Hoefert[18] and Gorke[19] found large numbers and varieties of bacteria in the duodenum of many patients with diseased bile ducts. Gorke[19] found 6 cases in which B. coli seemed to be present in pure culture.

N. and J. Mutch,[23] working chiefly on streptococci and their growth relations to B. coli, found an increase of absolute numbers and of varieties in pathologic conditions. They mentioned "the great frequency with which chronic streptococcal disease of the intestines exists in rheumatoid arthritis." They noted that extensive streptococcal infections of the small intestine may exist even when repeated cultivation of the feces fails to yield any growth.

OBSERVATIONS ON INFANTS, CHILDREN AND ANIMALS

Much of the early investigation on intestinal and fecal flora was carried out on infants and young children, the study of intestinal pathology in infants forming a specialized field.

Among those making important observations on the small intestine of normal infants and children were Escherich,[28] Tissier,[29] Sittler,[30]

[25] Arch. f. Verdauungskr., 1901, 7, p. 43.
[26] Deutsch. Arch. f. klin. Med., 1906, 87, p. 254.
[27] Berl. klin. Wchnschr., 1912, 42, p. 1985.
[28] Die Darmbakterien des Saüglings, 1886.
[29] Ann. de l'Inst. Pasteur, 1908, 22, p. 189.
[30] Die Wichtigsten Bakterientypen der Darmflora beim Saügling, 1909.

Moro;[31] Hess,[32] who was the first to examine directly the bacterial contents of the duodenum of infants during the natural processes of digestion; Sisson,[33] who followed his method, and Bessau and Bossert.[34]

Most of these observers also investigated pathologic conditions and looked for specific bacterial agents to account for the disease conditions. Their results were conflicting. Moro[35] and his school[36] are responsible for the conception of an endogenous infection with B. coli in acute dyspeptic conditions.

In attempting to interpret the conditions associated with acute or subacute gastrointestinal disturbances not initiated by one of the generally recognized specific infective agents, the problem has come under investigation of the importance of the rôle played by bacteria and of the causes that bring about a change, where such exists, in the relative bacterial freedom of the upper small intestine.

The limitations normally imposed on the flora have variously been ascribed to the single or combined action of four main factors: (1) the mechanical factor involving the rapid clearing of the small intestine through peristalsis; (2) the chemical factor involving the rate of absorption of food stuffs, and a reaction of the chyme influenced by the food metabolites and the fermentation products of an obligate flora; (3) a factor depending on the production of special inhibitory substances by the normal intestinal bacteria; (4) a factor depending on the bactericidal activity of the digestive juices or the intestinal epithelium, and the opposition offered by the mucosa to bacterial invasion. Researches, many of which have been carried on in association with studies on infant morbidity, have concerned themselves with an inhibitory action on two distinct classes of bacteria, namely, foreign organisms experimentally introduced and the native flora of the alimentary tract.

Attempts were made to introduce foreign bacteria into the alimentary system of animals and to follow their fate in the intestine or in the blood stream and other organs, by Schütz,[25] Kohlbrugge,[11] Ficker,[37] Rolly and Liebermeister,[13] Moro,[31] Seiffert,[38] and Kulka.[39] Some of these investigators, particularly Rolly and Liebermeister,[13] tried to determine whether any single or combined factors, such as the activity of the various digestive juices, or of the ground up intestinal mucosa were responsible for a bactericidal action of the small intestine. But

[31] Arch. f. Kinderh., 1906, 43, p. 340.
[32] Ergebn. d. inn. Med. u. Kinderh., 1914, 13, p. 530.
[33] Am. Jour. Dis. Child., 1917, 13, p. 117.
[34] Jahrb. f. Kinderh., 1919, 89, p. 213.
[35] München. med. Wchnschr., 1916, 66, p. 1134.
[36] Hauschild, L.: Ztschr. f. Kinderh., 1922, 31, p. 399.
[37] Arch. f. Hyg., 1905, 52, p. 179.
[38] Deutsch. med. Wchnschr., 1911, 37, p. 1064.
[39] Arch. f. Hyg., 1914, 82, p. 337.

while foreign bacteria were usually destroyed, they could not isolate any single factor to account for this effect.

Ganter and Van der Reis[20] reported that if they introduced B. prodigiosus at various points in the small intestine by means of their metal cylinders, controlled by a magnet, they could not recover the organism from the feces, but that cultivation was successful if the bacteria were introduced at some spot in the large intestine. They also found that if they introduced silk threads dipped into culture of B. prodigiosus into their cylinders, and then took up intestinal contents at various levels, again closing the cylinders as soon as they were filled, these threads, voided in the closed containers with the feces, yielded no B. prodigiosus when the cylinders were opened in the upper middle or lower small intestine, but yielded cultures of B. prodigiosus when they were opened in the cecum. This result was independent of the presence of food or of the type of diet. Since, however, the small intestine, according to these authors, is not sterile but has a native flora, they preferred the term of autodisinfection to autosterilization applied to the bactericidal activities of the small intestine. Van der Reis[21] later reported that in fermentative dyspepsia he found the autodisinfection ability of the small intestine much disturbed.

Various explanations also have been offered to account for the comparative bacterial freedom of the small intestine under ordinary conditions, i. e., the rapid passage of the chyme (Kendall,[15] Bessau and Bossert,[34]) successful competition of an autogenous flora (Sittler[30]) and absence of large quantities of unabsorbed sugar in the lower small intestine (Hahn, Klocman and Moro).[40]

N. and J. Mutch[23] found that the factors encouraging a pathologic increase of streptococci in the chyme were: (1) the presence in the mouth of centers for the production of glycophilic streptococci, (2) impaired digestion, (3) loci of stagnation in the intestine, and (4) diminished resistance of tissues generally or locally. The presence of carbohydrate they considered the most potent factor in determining the predominance of glycophilic streptococci over B. coli at any level.

Finally, reference should be made to the researches of Dragstedt and his co-workers[41] on closed intestinal loops in dogs and rats. They found that even where the accumulation of fluid with consequent toxemia was prevented, the loops contained a rich proteolytic flora. They, therefore, concluded that there was no evidence of a marked bactericidal action on the part of the intestinal juice so far as this group of organisms was concerned. They noted that necrosis greatly facilitated the absorption of toxins (they did not look for bacterial invasion), and they made the further important observation that although toxemia might at times be prevented, it was impossible to kill all the bacteria in a loop even when using antiseptics strong enough to sterilize a surgeon's hands. Their results are to be compared with those of Bloomfield[42] on the ineffectiveness of antiseptics in causing more than a transitory change of the bacterial picture of the upper respiratory passages.

Relation of Bacterial Flora to Food

Effect of Starvation.—Marfan and Bernard,[9] working on dogs, found that a 24-hour fast renders the duodenum amicrobal. Sisson[33] found that fasting induces a relatively amicrobal condition in the whole small intestine of puppies.

[40] Jahrb. f. Kinderh., 1916, 84, p. 10.

[41] Jour. Exper. Med., 1917, 25, p. 421; 1918, 27, p. 359; 1919, 30, p. 109; Jour. Infect. Dis., 1922, 31, p. 209; Jour. Lab. & Clin. Med., 1922, 8, p. 190.

[42] Bull. Johns. Hopkins Hosp., 1923, 34, p. 65.

Cushing and Livingood[10] found the scrapings from the lower limb of a jejunal fistula sterile, and believed that the small intestine of man could be rendered entirely amicrobal through fasting. Ficker[37] found that after starvation the B. coli flora of the small intestines of animals was richer than under normal conditions. Rougentzoff[43] noted an increase of anaerobes during starvation and the absence of acidophilic organisms. Dragstedt, Cannon and Dragstedt[41] found that acidophilic bacteria originally present disappeared from the closed intestinal loops of rats and dogs, probably because of the absence of suitable food, their place being taken by proteolytic organisms. Van der Reis[21] found that fasting lowered the number of bacteria in the small intestine of man, but did not bring about an amicrobal condition. Bogendorfer[28] also found in the human small intestine, after eating, an increase of viable organisms, chiefly of the streptococcus group.

EFFECT OF THE CHARACTER OF THE FOOD ON THE FLORA

In the course of the numerous studies that have been carried out on the effect of diet on the flora of the feces, certain observations on the alteration of the flora of the small intestine of animals have also been made. Herter and Kendall,[44] Torrey,[17] and Rettger and Cheplin,[45] all found that a carbohydrate diet favored a fermentative and acidophilic flora with elimination of putrefactive bacteria, while meat, according to Herter and Kendall,[44] and to Torrey,[17] favored the proteolytic organisms, very much as has been found to be the case in the feces. Sisson,[33] however, found little fundamental change with variations in diet.

Dragstedt, Cannon and Dragstedt[41] found that rats and dogs fed on a diet containing lactose previous to complete obstruction of the lower colon lived longer than those previously receiving a meat diet. Nevertheless, even in the animals that had received the lactose and had responded by the development of an aciduric flora, the final flora at death was proteolytic in the intestine above the point of obstruction. In closed isolated intestinal segments still attached to the mesentery the flora rapidly became purely proteolytic, irrespective of its original character.

Van der Reis[21] found that in man also an increased carbohydrate diet over an extended period of time augmented the milk souring and aciduric bacilli of the small intestine.

In concluding our survey of the literature, we may summarize the points on which there seems to be substantial agreement, and those about which controversy exists.

Most observers agree that under normal conditions there is a comparatively low bacterial count in the duodenum and jejunum, with a distinct increase in the lower ileum; that there is no marked preponderance of the colon group until the lower ileum or the cecum is reached; that putrefaction due to proteolytic bacteria is insignificant in the upper two thirds of the small intestine; that cocci are present in considerable if not preponderant numbers in this section; that the acidity of the

[43] Ann. de l'Inst. Pasteur, 1914, 28, p. 639.
[44] Jour. Biol. Chem., 1910, 7, p. 203.
[45] Intestinal Flora, 1921.

stomach probably influences the duodenal flora; and that absence of food reduces bacterial numbers.

The chief points concerning which there is considerable disagreement are the existence of an obligate indigenous flora independent of the bacterial features of the ingesta; the significance of the colon group and of pyogenic cocci when found in the upper small intestine and their etiologic importance in certain pathologic conditions; the existence of a bactericidal activity on the part of the intestinal epithelium; and the importance of various factors in preserving the normal bacterial conditions in the small intestine.

EXPERIMENTAL

Swallowing and retaining the tube was associated with considerable difficulty and at times discomfort. The patients varied greatly in their psychologic reaction to the experience. On the whole, the women were more amenable than the men. Many attempts met with failure. Sometimes the tube was retained for a certain number of hours and then withdrawn by the subject. It required great tact and patience on the part of the physician, as well as considerable intelligence on the part of the subject, to carry through a successful experiment.

Preparation of the Duodenal Tube (Dr. Murphey).—The required length of rubber tubing is sterilized by boiling for 15 minutes in water. The excess water is drained from the tube. The duodenal bucket is then passed a few times through a low gas flame, so as to remove excess water and also to render the outer surface sterile. A double zero (00) gelatin capsule which has previously been cut to the desired size and rendered sterile by preserving in 95% alcohol is then drained free of its alcohol and slipped over the now cool duodenal bucket, so that the upper edge of the capsule is just beyond the highest perforation on the bucket. Parvaseline which has previously been brought to the melting point in the ordinary type of spray apparatus used for the treatment of burns, is then sprayed gently onto the covered end of the bucket until the whole surface of the capsule and that portion of the bucket up to its junction with the rubber tubing, is covered with a thin intact layer of wax. This is allowed to cool and is then molded to the form of the bucket, paying special attention to the upper and lower limits of the wax while it is still plastic. The upper end of the rubber tube is provided with a stopcock, and when this is closed the inside of the rubber tubing is protected from contamination, provided the joints in the rubber tubing and the layer of wax on the bucket have been rendered watertight.

The tube was administered to the patient in the morning on an empty stomach. After the bulb had reached the stomach, he was instructed to lie on his right side for one and one half hours. From that time on, he was to swallow the tube slowly, about 1 inch every 10 minutes. On account of the softness of the rubber, it was difficult to get a grip on the tube with the muscles of the throat. Often the throat became quite sore. He was instructed to continue

feeding himself the tube until a point marked with a white thread was reached. He usually retained the tube about 24 hours.

In withdrawing a sample, air was forced into the tube under sufficient pressure to perforate the wax, thus opening up some of the perforations on the bucket. By means of ordinary syringe suction the material was then aspirated under as nearly aseptic conditions as was experimentally possible. It was collected in a sterile test tube, the withdrawing of sufficient material often taking considerable time.

During the day, unless specifically stated, the patients ate regularly. They also had a light breakfast at about 7 a. m., about two and one half to three hours before the sample was taken. Most specimens from the duodenum, according to the literature, have been taken after 12 to 14 hours' fast. The advantage of our method is that it is a test applied under the normal conditions of digestion. We get some idea of whether in the jejuno-ileac region the flora is as mixed as with the chance organisms introduced by the food we might expect it to be, or whether at this level it has already undergone a severe process of selection. On the other hand, the results are susceptible to great fluctuations, depending on whether the capsule is located at a point where there is or is not an accumulation of chyme. Difficulties at various times in aspirating material were undoubtedly in part due to clogging of the openings of the olive with paraffine, and in part to the absence of material at that particular point.

From the fact that a certain number of inches of the tube had been swallowed, it cannot of course be assumed that either in different persons or in the same person at different times a similar point in the intestine had been reached. Due to peristaltic action, the tube, when there are no coilings, is shorter than the length of intestine which it has traversed; that is, it cuts the corners of the intestinal loops. The degree, however, of the short-cutting will vary with conditions, such as the vigor of the peristaltic action and the distention of the intestine.

Two other points of importance should be noted. One is the probability that occasionally, as a result of the irritation due to the tube, a slight reversal of peristalsis may take place; the other, that following prolonged suction when fluid is not at once aspirated, a partial vacuum may be formed in the intestine, and material from a lower level may gradually rise up to the point where the olive is situated. There were several specimens which we judged might consist of regurgitated material swept into the tube by a reversed peristalsis.

After the sample had been withdrawn the patient was sent to the roentgen-ray room of Bellevue Hospital for a photographic record, after which a report on the position of the olive and of coils of the tube in the stomach, if such were present, was given to us. In a few cases only no confirmatory photograph was taken. A few photographs were taken at the Willard Parker Hospital and were seen by us. They demonstrated the fact that while there is often no uncertainty as to position of the olive, there may sometimes be difficulty in determining whether or not there are coils present in the stomach.

Methods of Cultivation and Analysis.—The technic of cultivating the specimens was modified and amplified during the research. The final method in its complete form is given here.

The specimen was brought to us in a sterile test tube immersed in ice. We kept it on ice until ready to make the dilutions, our object being to proceed as rapidly as was consistent with careful work. We then treated the material in the following manner.

UNHEATED MATERIAL

Pour Plates.—The material used was: Rettger's modification of Endo agar (lactose, bisulphite and fuchsin added just before pouring); citrated horse blood veal agar, P_H 7.6-7.8; acid beef liver agar (Torrey[46] P_H 5.8-6 with 1% dextrose and 1% lactose added just before pouring. Usually we made pour plates inoculated with 0.5 c c. of 1 : 10, 0.1 c c. of 1 : 10, 0.1 c c. of 1 : 100, or 0.1 c c. of 1 : 1,000 dilution. Streak plates of the undiluted material were also made on the different kinds of mediums mentioned.

Tubes.—1% dextrose beef broth fermentation tube P_H 7.6, 0.5 c c. of 1 : 10 dilution; sugar-free veal broth fermentation tube (neutral to phenolphthalein) 0.5 c c. of 1 : 10 dilution; litmus or brom cresol purple milk under anaerobic conditions 0.5 c c. of 1 : 10 dilution; cooked meat medium under anaerobic conditions 0.5 c c. and 0.1 c c. of 1 : 10, 0.1 c c. of 1 : 100, 0.1 c c. of 1 : 1,000, 0.1 c c. of 1 : 10,000, 0.1 c c. of 1 : 100,000 dilutions; $\frac{\text{normal}}{20}$ acetic acid veal broth (2% dextrose) 0.5 c c. of 1 : 10, 0.1 c c. of 1 : 10 dilutions; $\frac{\text{normal}}{10}$ acetic acid veal broth (2% dextrose) 0.5 c c. of 1 : 10 dilution; gelatin (stab) 1 drop of original material, 1 drop of 1 : 10 dilution.

ORIGINAL MATERIAL HEATED TO TEST FOR SPORES FOR 15-20 MINUTES AT 80 C.

(When enough material was aspirated)

Tubes of cooked meat medium under anaerobic conditions, 0.2-0.5 c c. of undiluted material, 0.5 c c. of 1 : 10 dilution, 0.1 c c. of 1 : 10 dilution, aerobic plate or series of broth tubes corresponding to tubes of cooked meat medium.

GRAM STAIN DIFFERENTIAL COUNT

All the material, except the gelatin tubes was incubated for from 18 to 20 hours at 37 C. and then examined and the colonies counted on the Endo and sometimes on the blood plates. The latter were always returned to the incubator. The rest of the material was incubated for another 24 hours, or if the sample was poured on Friday, the examination did not take place until Monday. Our procedure was as follows:

For making numerical estimates of colonies a counting plate was used. On the Endo plates we made a count, attempting to differentiate between colonies representing presumptively the colon group, lactose fermenting streptococci, and nonlactose fermenting gram-negative bacilli. If different types of colonies were present, we usually isolated strains in order to distinguish through sugar reactions between B. coli, B. lactis-aerogenes, and other gram-negative bacilli.

On the citrated blood veal plate, we looked specifically for hemolytic, green-producing, or gamma type streptococci and staphylococci, making a differential count whenever possible.

The high H-ion concentration of the acid beef liver medium was supposed to suppress many organisms, and to favor, because of the rich sugar content, the acidophilus group. The count, however, on plates of this medium was often high even in the absence of B. acidophilus, as most of the glycophylic streptococci grew well on it.

For some time dilution plates were also poured with beef liver agar P_H 7.8-7.9 containing 2.5% glycerol and 2% dextrose. This medium was recommended

[46] Jour. Bacteriol., 1917, 2, p. 435

by Kopeloff and Morse [47] for their method of anaerobic plate culture. Originally we plated our material on this medium and incubated it under aerobic and anaerobic conditions, but we found that with the great concentration of aerobes, the obligate anaerobes were usually overgrown or appeared only in symbiosis. We therefore abandoned the method, relying entirely on our cooked meat medium for the development of anaerobes.

Conditions in the dextrose and the sugar-free broth fermentation tubes are respectively favorable to different types of those organisms which are present in the original sample. We studied the sediments after 48 hours and returned the tubes to the incubator for further study after another 4 or 5 days. Sometimes different types of organisms were isolated by pouring or streaking plates from these sediments. Conditions in the dextrose broth fermentation tube favor certain strains of streptococci, staphylococci, yeasts and B. acidophilus, while those in the sugar-free broth fermentation tube favor, through suppressions of glycophylic bacteria, the colon group, B. proteus and spore-bearing aerobes.

The oxygen tension in the milk tube was reduced by heating in boiling water for about 5 minutes and adding a petrolatum layer of about ½ inch. We looked for gas and acid formation, noted the type of clot, when one had formed, and observed whether gradual peptonizing took place. The tube was kept for at least one month, noting macroscopic and microscopic changes. If there was much gas we sometimes made a series of rapid transplantations in milk with the object of favoring B. welchii, if present.

The tubes of cooked meat medium were autoclaved with a petrolatum plug of from ½ to 1 inch. We carefully observed them for a period of at least one month, noting the following points: gas formation, digestion, swelling, blackening or reddening of meat, and changes in the character of the gram-stained film. The object was to determine the highest dilution containing anaerobes, but we did not always succeed in this, although we often established the presence of anaerobes in one of the tubes. We transplanted from the original tube into a second cooked meat tube and into a dextrose broth tube. If the film from the second cooked meat tube showed bacilli resembling the well-known types of the spore-bearing anaerobes, when no such organisms could be found in the aerobic tubes, we regarded this as a presumptive test indicating the presence of anaerobes. We then tried to isolate the organisms. When both spore-bearing aerobes and anaerobes were present, the presumptive test was of no value, as similar forms were found in both the anaerobic and aerobic tubes. Toward the end of our work we adopted Hall's [48] method of separation by inhibiting the aerobes with a dilute solution of methyl violet. We found the technic valuable in some cases.

By a series of tubes we also estimated roughly the number of spores present in our heated material. Here too we found the liquid mediums more satisfactory than the anaerobic plates. Simonds [49] found that the presence of 2 or 3 welchii spores is sufficient to give a stormy reaction in milk, and we arbitrarily assumed, in making our estimate, that a single spore will develop in the cooked meat tube.

The tube of N/10 and N/20 2% dextrose acetic acid broth were examined for aciduric organisms, especially those of the B. acidophilus group.

In general, the observations which hold for any mixed culture are particularly applicable to the intestinal contents. It is easy from plat-

[47] Am. Jour. Pub. Health, 1922, 12, p. 119.
[48] Jour. Am. Med. Assn., 1919, 72, p. 274.
[49] Monograph Rockefeller Inst. for Med. Res., 1915, 5.

ings alone to get a false impression of the dominant organisms; or, if the dilutions used in the pour plates are not sufficiently high, a majority organism may be entirely suppressed. We once found in a specimen that 98% of the aerobes consisted of delicately growing green streptococci, which were completely inhibited on the more crowded plates. On the other hand, the B. acidophilus group is highly symbiotic, so that in a mixed culture a far greater percentage of colonies will appear on a crowded than on a thinly settled plate. Torrey [50] noted the tendency of B. acidophilus colonies to form a satellite grouping around a B. coli colony. In broth, again, an organism which seems to form few colonies on the pour plates may appear in very high dilutions. In general, we may say that pour plates, even when only a few colonies appear on the highest dilutions, probably always give too low an estimate of numbers. We are convinced that many bacteria which might grow in a liquid medium are too feeble to develop colonies on a solid medium. In order to do justice to a specimen, it should be subjected to a severe and varied process of analysis. In some cases we feel that we have approached this ideal, but often we realize that our results sketch an incomplete picture.

NORMAL PERSONS

The jejunal contents were obtained from 4 apparently healthy persons (3 women and 1 man), who volunteered to swallow the tube. They ranged from 21 to 53 years of age. The tube length varied from 40″to 65″. In one case, that of Fr., 2 specimens were taken. The first one, which was aspirated with difficulty, appeared clear and colorless. The second specimen, taken immediately afterward, at a level about 10 inches higher up, was aspirated with greater ease, and had the usual yellowish bile color. This is one of the cases referred to above as a possible instance of reversed peristalsis. The count on the second specimen was almost 20 times as high as that on the first specimen. Both portions were studied, and except in respect to bacterial numbers, gave almost similar results. Only the first specimen, however, is recorded in table 1.

Table 1 summarizes the salient features of the specimens from normal persons. The following points are to be noted:

1. The limits of bacterial concentration are far apart, ranging from less than 100 to over 100,000 bacteria per c c.

[50] Jour. Infect. Dis., 1915, 16, p. 72.

TABLE 1

SUMMARY OF THE RESULTS ON THE INTESTINAL CONTENTS OF FOUR NORMAL PERSONS

Subject	Age	Depth of Tube from Teeth	Highest Bacterial Counts per C c. on Aerobic Pour Plates	Medium Showing Highest Bacterial Count	Gas in Dextrose Broth Fermentation Tube	Spores in Sugar-Free Broth Fermentation Tube	Spores Developing in Undiluted Material 0.2-0.5 C c.	Peptonizing of Milk	Proteolytic Activity in Cooked Meat Broth Medium	Sporing Aerobic Bacilli	Anaerobic Bacilli	Gram-negative Bacilli	Streptococci or Diplococci	Gram-positive Non-sporing Aciduric Bacilli
Ban	21	50″	1 4 0	Blood veal	—	+	++	+	+	++	—	++ (colon group)	+	—
R.	53	65″-70″	2 9 0 0 0 0	Acid beef liver	++	+	—	+	+	×	—	Strongly predominant (colon group)		—
C.	21	60″	2 0 0 0 0	Count on blood veal very much higher than on other mediums	—	+	+	+	+ (On surface of meat column)	+	—	—	Strongly predominant (gamma streptococci)	—
Fr.	28	50″	7 0 0 0 0	Blood veal	+	+	....	—	—	+	—	Predominant (colon group)	++	×

In tables 1 and 2, the following symbols are used: ++, present in large numbers or amounts; +, present; ×, presence observed, but not definitely confirmed by isolation of organism; —, not present as far as observed.

TABLE 2

SUMMARY OF RESULTS ON THE INTESTINAL CONTENTS OF TWENTY HOSPITAL PATIENTS

Subject		Age	Depth of Tube from Teeth	Diagnosis; Symptoms; Diet	Highest Bacterial Counts per C c. on Aerobic Pour Plates	Medium Showing Highest Bacterial Count	Gas in Dextrose Broth Fermentation Tube	Spores in Sugar-Free Fermentation Tube	Spores Developing in Undiluted Heated Material 0.2-0.5 C c.	Peptonizing of Milk	Proteolytic Activity in Cooked Meat Medium	Sporing Aerobic Bacilli	Anaerobic Bacilli	Gram-Negative Aerobic Bacilli	Streptococci, Diplococci or Sarcinae	Gram-Positive Non-sporing Aciduric Bacilli
O'M.	♂	44	30″ approx.	Carcinoma of right lung; constipation; regular diet	6 1 0 0	Endo	+	—	Not tested	+	Not tested	—	+	++	+	—
M.	♂	43	30″ approx-	Syphilitic; regular diet at time of taking specimen	5 5 0 0	Veal	Not recorded	—	Not tested	—	Not tested	+	—	+	+ gamma streptococci	+
Kom.*	♀	22	40″ approx.	Erythema nodosum following sore throat; soft special diet (stomach not empty when tube was passed); poor appetite	4 4 0	Endo	—	No growth	Not tested	—	Not tested	—	—	—	—	—
K	♀	55	75″	Influenza; chronic interstitial nephritis; soft special diet	1 7 0 0 0	Acid beef liver	+	—	Not tested	—	+	×	×	+	+	+
Cr I	♀	24	80″ saline washing	Phlebitis following childbirth, embolic lobar pneumonia, right-sided hemiplegia, chronic constipation; regular diet	5 0 0 0 0 0	Acid beef liver	+	—	Not tested	—	+	—	+	+	Predominating gamma and alpha streptococci	—
Cr. II		..	70″	.....	1 0 6 0 0	Acid beef liver	Not recorded	+	--	+	+	+	—	—	Predominating gamma and alpha streptococci	+
T.	♀	24	70″	Chronic pulmonary tuberculosis, gaseous eructations; soft special diet	....	All plates sterile	Contaminated	No growth	Not tested	No growth	No growth	—	—	—	—	—
P.	♀	44	65″ roentgen-ray report: 2d part of duodenum	Influenza-neurasthenia, hysterical gastric manifestations; soft special diet	5 0 0	Endo	No growth	—	+	—	—	—	—	—	—	—
J I.	♀	38	70″	Bilateral pyelonephritis, cystitis, no calculus; low protein diet	4 3 0 0 0 0 0	Endo	+	—	+	+	++	+	++	Predominating among aerobes	+ Alpha streptococci	+

* Only yeast developed from this specimen.

J. II		..	75″ saline washing coils in stomach	.....	4 3 0 0 0 0	Acid beef liver	+	—	—	—	Not recorded	±	—	++	+	×
J. III		..	90″	.....	2 1 6 0 0 0	Acid beef liver	Contaminated	—	+	—	±	—	+	++	×	—
Sp.	♀	25	40″	Neurasthenia, sciatic pain; special soft diet	.....	All plates sterile	—	+ Possibly contaminated	Not tested	No growth	No growth	±	—	—	—	—
Meach.	♀	57	80″	Charcot's joint, cardionephritis, poor appetite; soft diet	1 6 0 0	Blood veal	—	—	Not tested	—	+	—	×	—	++ Alpha streptococci	×
Jol.	♀	48	80″	Pernicious anemia, roentgen ray shows retention and slight delay of pyloric opening; soft special diet	3 2 0 0 0 0 0 0	Blood veal and acid beef liver	Not recorded	—	—	—	—	—	—	Predominating (colon group)	+ Gamma streptococci	+
H.	♀	21	65″	Gonorrheal arthritis; regular diet	2 0 0	Blood veal; no growth on acid beef liver and endo	—	—	—	No growth	+	—	—	—	Cocci in sarcinae-like groupings completely predominating	—
V. I †	♀	48	75″	Severe anemia; operation probably for carcinoma of stomach with gastrojejunostomy; soft special diet	5 0 0 0 0 0 0 0+	More than 50 million bacterial count on all plates	+	—	++	—	++	++	×	++	—	+
V. II		..	80″–90″ approx.	.....	1 2 0 0 0 0 0 0	Blood veal	+	+	++	++	±	++	++	++	+ Alpha streptococci	+
V. III		..	80″	.....	5 4 0 0 0 0 0	Endo and acid beef liver	++	—	-	—	+	—	—	Predominating (colon group)	+	+
Feld. I	♀	29	70″	Addison's disease; soft special diet	8 8 0	Blood veal	+	—	—	—	+	—	±	Predominating	—	—
Feld. II		..	65″	.....	2 1 0 0	Blood veal much higher than endo or acid beef liver	—	—	Not tested	—	—	—	—	—	Predominating, gamma streptococci	—
Br.	♀	47	45″ approx. saline washing	Arthritis deformans, occasional vomiting; no food taken during 24 hours of retaining tube; soft special diet	.....	Endo (yeast predominant organism)	—	—	—	No growth	—	—	—	+ Not of colon group	+	—
Sh. I	♀	25	30″	Articular rheumatism; soft special diet	3 2 0	Dilution tubes of cooked meat broth (anaerobic)	—	+	+	++	++	++	Predominating, more than 100,000 per c c.	+	+	—

TABLE 2—*Continued*

SUMMARY OF RESULTS ON THE INTESTINAL CONTENTS OF TWENTY HOSPITAL PATIENTS

Subject	Age	Depth of Tube from Teeth	Diagnosis; Symptoms; Diet	Highest Bacterial Counts per C c. on Aerobic Pour Plates	Medium Showing Highest Bacterial Count	Gas in Dextrose Broth Fermentation Tube	Spores in Sugar-Free Fermentation Tube	Spores Developing in Undiluted Heated Material 0.2-0.5 C c.	Peptonizing of Milk	Proteolytic Activity in Cooked Meat Medium	Sporing Aerobic Bacilli	Anaerobic Bacilli	Gram-Negative Aerobic Bacilli	Streptococci, Diplococci or Sarcinae	Gram-Positive Non-sporing Aciduric Bacilli
Sh. II	..	65″	.....	1 0 5 0 0 0	Blood veal much higher than endo or acid beef liver	—	++	++	++	+	++	—	—	++ Alpha streptococci	—
A. ♀	21	45″	Chronic arthritis; history of rheumatic fever; soft special diet	2 8 0 0 0 0	Blood veal	+	++	+	++	++	+	++	++	+	+
Ck. ♂	32	60″ approx.	Pellagra, diarrhea; had eaten little for several weeks; diet: meat, potatoes	1 6 9 0 0	.....	+	+	—	—	—	+	×	—	+ Alpha and gamma streptococci	—
B. ♂	23	60″ roentgen ray report: first part of duodenum	Ischiorectal abscess, previous history of acute endocarditis	5 4 0 0 0	Blood veal much higher than endo or acid beef liver	—	—	—	+	+	—	Probably a few ±	Very few	Completely predominating gamma streptococci	—
W. I ♀	42	80″	Arthritis deformans, constipation; soft special diet	9 6 0 0 0 0	Blood veal	+	+	—	++	—	×	—	+	Predominating alpha and gamma streptococci	+
W. II	..	90″	.....	2 8 2 0 0 0	Blood veal	Not recorded	—	—	+	Not recorded	—	±	+	Predominating alpha and gamma streptococci	+
W. III	..	35″–40″ approx.	.....	2 9 5 0 0 0	Acid beef liver	—	—	Not tested	—	+	—	++	±	Completely predominating among aerobes alpha and gamma streptococci	+
W. V	..	90″ approx.	.....	1 0 0 0 0 0 0	Blood veal	+	+	Not tested	+	+	++	—	++	++	+
W. VI	..	70″–80″ approx.	.....	3 2 4 0 0 0 0	Acid beef liver	+	—	++	+	++	±	++	+	++	×

† This specimen yielded over fifty million bacteria per c c. It was fecal in appearance. Probably a case of reversed peristalsis.

2. The two cases in which the flora seemed most varied gave, respectively, the highest and the lowest bacterial count.

3. Only two of the specimens developed gas in the dextrose broth fermentation tubes; the production of gas seems directly associated with the presence of large numbers of gram-negative bacilli.

4. Spores were present in the sediments of all of the sugar-free broth fermentation tubes.

5. Proteolytic activity evidenced by peptonizing of milk and digestion of meat, was found in 3 out of 4 cases. In the case of C., the digestion in the cooked meat medium was slight, taking place only on the top layer of the meat.

6. Anaerobes could not be found in any of the specimens, although a special search was made for them. In view of the presence of spore-bearing aerobes, it seems probable that these organisms were responsible for the proteolytic manifestations. In one case, that of R., the sporulating aerobe was not isolated, and we are not certain that anaerobes of which the growth was suppressed by the preponderant gram-negative bacilli may not have been present in small numbers.

7. Gram-negative bacilli of the colon group were present in large numbers in the specimens of 2 persons and predominant in the specimen of a third person with a low count.

8. Gram-positive streptococci or diplococci were regularly present, once as the preponderant organisms. We should note, in contrast to later findings recorded of abnormal cases, that green producing streptococci were not observed.

HOSPITAL CASES

These specimens were obtained from patients in the medical wards of Bellevue Hospital; one specimen, that of B., came from a patient in the surgical ward. We record results on 20 patients. Their ages range from 21 to 57 years. Some of the results of our analyses are recorded in table 2. This table will be discussed in detail later.

In making any comparisons with normal persons, it is of course necessary to consider that the condition from which some patients were suffering had no relation to disturbance in the gastro-intestinal tract, while in the condition of others there may have been such a direct relation, or there existed the possibility of a bacterial focus of infection in the alimentary tract. In going over the case histories, it is found that many of the patients showed symptoms of chronic consti-

pation, but as such a condition may be found among many "normal" persons, we need not specifically consider this factor. It is interesting to study the relation of the bacterial numbers to the case history. This point is taken up in table 3.

We note that in the first column in which are listed the diseases of those persons whose specimens gave less than a count of 10,000 bacteria per c c., no condition is found which we might associate with disease of the gastro-intestinal tract or with a possible focus of infection in the

TABLE 3

SUMMARY OF THE RELATION OF THE BACTERIAL COUNT TO THE CLINICAL DIAGNOSIS

Approximate Depth of Tube	Clinical Diagnoses of Patients With Specimens Having			
	Less Than 10,000 Bacteria per C c.	10,000–100,000 Bacteria per C c.	100,000–1,000,000 Bacteria per C c.	More Than 1,000,000 Bacteria per C c.
25″-40″	Carcinoma of right lung (1) Erythema nodosum (1) Neurasthenia, sciatica (1)	Ischiorectal abscess, previous history of acute endocarditis (1)	*Articular rheumatism (1) Syphilis (1)	
40″-60″	Arthritis deformans (1)	Pellagra (1)	Chronic arthritis (1)	
60″-80″	Addison's disease (2) Gonorrheal arthritis (1) †Gastritis, probably of hysterical origin (1)†	Influenza, chronic; interstitial nephritis (1) Charcot's joint (1) *Articular rheumatism (1) *Phlebitis (1)	*Phlebitis (1) *Cystitis (1) *Arthritis deformans (1)	*Cystitis (1) Pernicious anemia (1) *Anemia (gastro-jejunostomy (2) *Arthritis deformans (1)
More than 80″	.....	....	*Cystitis (1) *Arthritis deformans (2)	*Anemia (gastro-jejunostomy (1)

The numerals next to the diagnosis refer to the number of specimens taken from one patient which fall into the same group.

* Wherever identical diagnoses are marked with a star it indicates that the specimens though falling into different groups are derived from the same patients.

† The specimen from this patient may have come from a higher level. The roentgen-ray report left this point in doubt.

intestine, except one case of arthritis deformans. This specimen was taken after a 24-hour fast and was a saline washing, so that the conditions are scarcely comparable to those under which most of the specimens were taken. The absence of food is especially likely to reduce the bacterial count. The gastritis of another patient was of a neurotic nature, hysterical manifestations masking all the others, and there was no reason to assume the presence of any genuine lesion. It is also true that several of the persons in the first column are among those who swallowed a comparatively short tube, or had most of the tube coiled

in the stomach, so that their specimens come from the lower duodenum or upper jejunum; this, however, is not uniformly the case, while some persons in whose intestines the olive did not descend very far, had specimens falling into columns 2 or 3.

In the third and last columns, however, consisting of cases giving specimens ranging between 100,000 and more than 1,000,000 bacteria per c c., we note 6 cases of diseases which are usually associated with abnormal gastro-intestinal conditions, or which have frequently been traced in medical literature to foci of infection in the intestinal tract.

There is one case of pernicious anemia and another of severe anemia possibly of a pernicious character. The history of this patient (V.) was somewhat obscure. The roentgen-ray, however, indicated a gastro-jejunostomy, and there was suspicion of a carcinoma of the stomach, although the patient stated that she had been operated on for gallstones. In both cases of anemia all specimens had counts of over 5,000,000 bacteria per c c. One suspects here the influence of hypo-acidity.

In the third column are two cases of arthritis, one deformans, and one chronic with a history of rheumatic fever, a disease which, according to Norman and Eggston [1] and to Mutch [2] may be accompanied by foci of infection in the intestinal tract. Similarly situated secondary foci have been associated in the literature with articular rheumatism, of which there is also a case, significant because of the high anaerobic count.

Finally, there is a case of cystitis, apparently of renal origin, with no evidence of calculus. One exploration of the left ureter yielded large streptobacilli and streptococci. On another occasion, colon-like bacilli were found in the specimens from both ureters. A common theory to account for bacterial infection of the pelvis of the kidney, when there is no primary involvement of the bladder, assumes the passage of the living bacteria through the intestinal wall to the kidney, either directly or through the circulatory system. There is good reason to believe that a condition of the mucosa which favors penetration by bacteria, especially of a proteolytic variety, is also one that will offer an advantageous environment for multiplication in the intestinal canal. We, therefore, regard with great interest the large numbers and varieties of bacteria found in this patient (J.) and the predominance of gram-negative bacilli of several species, many of them gelatin liquefiers, including a number of the B. proteus group.

Two of the diseases in the third column, i.e., syphilis and phlebitis, we cannot associate with specific infection of the intestinal canal.

The diseases listed in the second column are of a kind, such as carcinoma of the left lung, that seem entirely unassociated with gastro-intestinal lesions, or such as chronic interstitial nephritis, which have often been ascribed to toxic substances of alimentary tract origin. They represent a heterogeneous grouping.

While these cases are too few to permit generalization, nevertheless the groupings in columns 1, 3 and 4 seem suggestive. We must, however, not fail to note that one of our normal persons had a specimen giving a count of over 100,000 and that all but one of the normal controls gave a count of over 10,000 organisms per c c. On the other hand, dietary conditions of the normal and pathologic cases are for the most part not comparable. Most of the patients, it will be seen, were supplied with the soft special diet, a regimen from which meat and the heavy vegetables were excluded, and the food was of an easily digestible character.

The normal subjects were leading an active life, doubtlessly associated with a fuller and more variet diet, which contained meat and other heavier foods. Moreover, the two patients with specimens listed in column 3, whose diseases seemed entirely independent of possible gastro-intestinal complications (Cr. and M.), were receiving the regular hospital diet, which was nearer to that of a healthy person leading an active life. For that reason it seems fair to us to place some weight on the evidence derived from table 3 as to the possible diagnostic significance of a high bacterial count when the diet is controlled.

In table 4 the bacterial counts are arranged according to the depth at which the specimens were taken. By far the greater number were drawn with a tube length of over 70″. There are considerable difficulties involved in tabulating according to the depth, because the known tube length swallowed would often seem to place the olive at a different point from that given by the roentgen-ray report. Unless, however, the report stated the presence of coils in the stomach, the tabulation has been made according to tube length. In 5 cases in which only the roentgen-ray report and not the tube length has been recorded, we have, of course, judged the approximate depth from the roentgen-ray report. Several times the roentgen-ray report stated that the tube was in the ileum. As the jejunum is supposed to be about 95″ long, and the tube length swallowed was never more than

90″ to 100″, it seems most unlikely, even allowing for cutting of corners, that a point as far down as the ileum could have been reached. In spite, however, of these irregularities we seem justified in concluding that between the lower duodenum and the lower jejunum different persons appear to vary too much from one another in respect to bacterial numbers for us to be able to trace any constant gradual increase in numbers as we progress along this portion of the small intestine. All specimens, however, taken with a tube length of over 70″ had more than 10,000 bacteria per c c. Great increases in bacterial numbers do not, according to the literature, take place until a point near the ileococecal valve is reached.

TABLE 4

SUMMARY OF THE RELATION OF THE BACTERIAL COUNT TO THE DEPTH OF THE TUBE

Approximate Depth of Tube	No. of Specimens Having 0–100 Bacteria per C c.	No. of Specimens Having 100–1,000 Bacteria per C c.	No. of Specimens Having 1,000–10,000 Bacteria per C c.	No. of Specimens Having 10,000–100,000 Bacteria per C c.	No. of Specimens Having 100,000–1,000,000 Bacteria per C c.	No. of Specimens Having More Than 1,000,000 Bacteria per C c.
25″–40″	1	1	1	1	2	0
40″–50″	0	1	0	0	1	0
50″–60″	0	0	0	1	0	0
60″–70″	1	3†	1	2	1	0
70″–80″	0	0	0	1	3	4
More than 80″	0	0	0	0	3	1
Total	2	5	2	5	10	5

In two specimens in which the anaerobic count was found to be higher than the plate count (Meach., Sh. I) the total bacterial count was considered.

† One of these specimens, that of P., may have come from a higher level as the roentgen-ray report, while mentioning no coils in the stomach, states that the olive was in the second part of the duodenum.

Let us now return to table 2. In looking over these results, it must be kept in mind that a negative sign in a column listing classes of bacteria does not necessarily imply the absence of this group, as it may at any time have been overlooked. This statement applies particularly to the anaerobic bacilli and among the earlier specimens to the B. aciodphilus group. Nevertheless, a particular search was made for anaerobes, so that, had they been present in large numbers, the presumptive test, at least, should have been positive.

From 6 persons 2 or more specimens were taken. If we compare the samples from the same persons, we find that they correspond fairly well in regard to bacterial numbers. The estimates on the aerobic plates always place the bacterial counts in the same or adjoining columns,

except in the case of Sh. and here, regard for the high anaerobic count also places these 2 specimens in adjoining columns. When we consider the many factors that might lead to variation, this point would seem to confirm the reliability of the method. When we compare bacterial types, we find that B. coli were predominant, or present in large numbers, in all specimens of J. and V., that streptococci were predominant or numerous in all specimens of W. and both specimens of Cr.; but that in the case of Fld., at approximately the same level, the colon group was once, and the streptococcus group at another time, predominant, while in the case of Sh. the anaerobes and spore-bearing aerobes were most numerous in one specimen, and the streptococcal and spore-bearing aerobic groups predominant in a second specimen, the anaerobes apparently being absent. These two samples were, however, taken at levels supposed to be 35″ apart. Thus we find that no bacterial type is of necessity absolutely characteristic of the jejuno-ileac level, even in the same person, and that the condition is not entirely different from what it is in the rectum, in which we have found that B. coli, streptococci, or even B. acidophilus, may at different times be predominant in persons eating an ordinary mixed diet.

There was some difficulty in determining whether a specimen showed a marked supremacy of one bacterial type, or a varied flora, because, on the one hand, on account of the selective mediums, minority organsims might appear prominent, while on the other hand, delicately growing forms that were really numerous, might be suppressed.

We find that 4 of 6 specimens that showed the exclusive or nearly exclusive presence in the cultures of one bacterial type also had a count of less than 1,000 per c c. Only one specimen with a decidedly high count (B.—50,000 bacteria per c c.) yielded a practically homogeneous flora. The predominance of one species in no way prevents the survival of other possibly antagonistic species. This is a point we wish particularly to emphasize. For instance, in the specimens of J. and V., we found very large numbers of the colon group, and in that of J. also of B. proteus; nevertheless, in both these cases, the B. acidophilus group was fairly prominent. These bacilli appeared on the acid beef liver plates, in the dextrose fermentation tubes, or in the milk. Thus, a condition similar to that of the normal feces is approached.

Among the normal subjects, one-half of the specimens seemed completely dominated by one bacterial type. This was, however, not the case in the sample with a very low count.

Two of 4 normal, and 10 of 24 hospital specimens developed no gas in dextrose broth fermentation tubes. Of these 10, five were associated with a low count or no growth. Of the remaining 5 samples, there were 2 (Fld. II and B.) in which streptococci completely prevailed, 2 (Sh. 1 and II) showing a distinct predominance of proteolytic organisms (spore-bearing aerobes and anaerobes) and of streptococci, and one (W. III) showing a predominance of streptococci with the presence of many anaerobes.

Gram-negative bacilli were found in 75% of the cases. Many of them belonged in the colon group. Details of their cultural characteristics will be recorded in another section.

Gram-positive diplococci or streptococci were present in 84% of the cases. In one case, a specimen yielded a pure culture of cocci in sarcinae-like groupings. The cocci will also be discussed in a separate section; we now wish, however, to call attention to the frequency with which streptococci producing green discoloration on blood plates appeared in the specimens of our hospital cases.

Gram-positive aciduric bacilli of the B. acidophilus group were found in 49% of the cases. When it is considered that in some of the earlier specimens we may have overlooked these bacilli, it seems fair to assume that they are nearly as common at this level as the gram-negative bacilli of the colon group, although probably not present in as great a concentration.

Spores either were present in the original heated material, or developed to some extent in the sugar-free broth fermentation tubes in about 38% of the specimens from hospital patients. In a number of cases there was too little material for heating. Usually when growth took place in the original heated fluid, the number of spores was estimated as under 20 per c c. In 2 of the 3 specimens of V., and in the 2 specimens of Sh., however, it was estimated that more than 100 spores were present per c c. in the original sample. In the specimen of V. II, we estimated the presence of over 200 spores per c c. In only 22% of the samples were they found in the sugar-free broth fermentation tubes. As they also appeared in the sediments of the sugar-free broth fermentation tubes developed from our 4 normal specimens, we must conclude that their mere presence in the fermentation tube is not an unfavorable symptom. They usually seem to be spores of aerobic bacilli, and we ascribe their presence in the specimens of the normal subjects to the fact that these persons were regularly on an ordinary

diet containing meat, which is known to favor the proteolytic spore-bearing forms. Most of the patients, however, as we have noted, were over a considerable period of time on a soft special diet from which meat was excluded. This was the case with A., V., Sh. and W., all of whose specimens developed spores in the sugar-free broth tube. It may, therefore, well be that when the diet is poor in proteins of mammalian origin, the indications of proteolytic activity in the sugar-free broth tube, and also the presence of relatively large numbers of spores in the heated material, assume significance.

Milk was partially peptonized by 42% of our pathologic and by 75% of our normal specimens. In spite of the reduced oxygen tension in the milk tubes, we found that aerobes of the B. mesentericus group were usually responsible for the most complete peptonizing. Sometimes, as in the specimen of Cr. II, peptonizing was begun and then inhibited by the final overgrowth of B. acidophilus, the reduced oxygen tension being favorable to the development of this organism. Experience with the feces of normal persons inclines us to the opinion that marked peptonizing is quite as frequently produced by material from the middle small intestine as by the feces. Among our 31 pathologic specimens, 11 of the 13 that peptonized milk had a high bacterial count.

As regards evidence of some proteolytic activity in the cooked meat tubes, such as swelling with partial disintegration and digestion of the meat, we note that it is extremely common, occurring in about from 75% to 80% of pathologic and normal cases, respectively. Sometimes we found what resembled a gnawing away of the top layer of the meat, without any signs of activity in the depths, but for the most part the changes took place throughout the whole mass, and were at times accompanied by white crystal deposits. Often, the proteolytic activity was fairly well marked, when repeated efforts to isolate an anaerobe not only were unsuccessful, but yielded no indications beyond the appearance of the meat itself that such an organism was present. Several times, however, a spore-bearing aerobe was revealed. The lowest dilution in the cooked meat tubes was 0.5 c c. of a 1 : 10 suspension. Whether in the presence of the diluted digestive intestinal juices, facultative aerobes, such as certain members of the colon grroup, B. proteus, staphylococci, or sarcinae, might produce proteolytic effects, is a point worth investigating. We found that a drop of sterile intestinal contents was often sufficient to liquefy a tube of gelatin, and we noted that both when no anaerobes appeared to be present and also when they were

present in large numbers in the higher dilutions, the proteolytic activity in the series of tubes became progressively less marked, a fact seeming to indicate that vigorous proteolysis was not necessarily associated with anaerobes alone. In the material cultured from the specimen of the patient H., nothing could be found except various species of sarcinae. Some sarcinae are gelatin liquefiers, and in the presence of the intestinal enzymes, they appear to have digested one half of the column of meat. While in the milk tubes fermentative processes on the part of the bacteria will be favored, proteolytic activities will more easily develop in the cooked meat tubes. Thus we might account for the more frequent evidences of digestive processes in the latter mediums.

In spite of the favorable conditions for anaerobic growth in the cooked meat tubes, we found this medium also most useful for isolating spore-bearing aerobes. Certain species, such as the highly proteolytic B. mesentericus and B. cereus, grew excellently in this environment in symbiosis with anaerobes (Sh.). Inoculation of the tubes with pipet material probably introduced some oxygen. Spore-bearing aerobes were isolated, or definitely appeared to be present, in about 40% of the specimens from pathologic cases, while they appeared in all of the normal samples. As previously stated, we are inclined to ascribe the divergent results to differences in diet.

Anaerobes were isolated, or definitely appeared to be present, in about 42% of the specimens from pathologic cases, and seemed definitely absent in about 50% of the cases. We were doubtful about 3 specimens. In none of our normal samples could they be demonstrated. Without wishing, in the absence of more cases, to ascribe great significance to this distinction, we nevertheless wish to emphasize the point that the mixed diet of our normal subjects, apparently favorable to spore-bearing aerobes, should have been more encouraging to anaerobes than the diet in most of our pathologic cases, had other conditions at this level of the intestine been advantageous.

Anaerobes were present in considerable numbers in specimens from 2 of the 3 cases of arthritis, the 1 case of articular rheumatism, the 1 case of cystitis and 1 of the 2 cases of severe anemia. It is a point of interest that they were the predominant organisms in a specimen of Sh. (articular rheumatism) taken at a depth of only 30″, while in a later specimen taken at a depth of 65″ they could not be found.

One more point to note is the fact that while usually the blood veal plate yielded the highest count, nevertheless the acid beef liver plate

had a count that was often only slightly less high. The reaction of this medium was P_H 5.8-6, and therefore might be expected to inhibit many organisms. It was, however, rich in sugar (1% dextrose and 1% lactose). In 2 of our cases (*C*, normal; *B*, ischiorectal abscess) in which the gamma type streptococci or diplococci were strongly predominant, the count on the blood veal plates (P_H 7.6) was 50 to 60 times as great as on the acid beef liver plates, while several other specimens having a predominance of streptococci both of the gamma and green-producing variety yielded the highest colony count on the acid beef-liver plates. The acid tolerance of some of these streptococci was further demonstrated by their occasional presence in N/10 acetic 2% dextrose broth. They resembled normal green-producing fecal streptococci, many of which are also strongly acid resistant. In the specimen of V. the counts on the blood veal and acid beef-liver plates were close, although the many gram-negative bacilli of the colon group, the presence of large numbers of spore-bearing aerobes, and of anaerobes, and the rapid peptonizing of milk, suggested a "putrefactive" specimen. The conclusion that must be arrived at from such observations is that the bacterial character of the small intestinal contents is often highly mixed, and that its flora is adaptable to wide environmental variations.

THE GROUP OF GRAM-NEGATIVE BACILLI

I have at various points referred to the conflicting opinions that have been expressed as to the significance of B. coli in the duodenum and in the rest of the upper small intestine. Different investigators have attempted to ascribe an etiologic relationship to their presence in large numbers in pathologic conditions such as biliary diseases, infantile dyspepsia, etc.

Recent work in connection with water analysis has brought out the fact that the group of gram-negative bacilli is extremely heterogeneous, and that much careful work is necessary before an organism can be definitely classified. Levine [51] who gives the following definition of the colon group: "The colon group will, therefore, be considered to include non-sporing gram-negative bacilli which ferment lactose with the production of acid and gas and which are capable of growing aerobically" has developed the method of correlation of characteristics first suggested by Winslow, Kligler and Rothberg [52] to distinguish between

[51] Iowa State College of Agriculture and Mechanic Arts Official Publication, 1921, Bull. 62.
[52] Jour. Bacteriol., 1919, 4, p. 429.

members of the B. coli and the aerogenes group, in place of dependence on the positive or negative character of a single reaction.

We did not enter into a sufficiently exhaustive analysis of the strains isolated for definite classification, but the following observations can be recorded.

The majority of the strains isolated gave the characteristic B. coli type of colony on Endo plates; flat, deep red, with a greenish metallic luster, rather than the more convex lighter colony of the aerogenes group lacking the metallic luster.

Five of 35 strains tested gave a positive Voges-Proskauer reaction. These were distributed among the specimens of 3 of 12 persons.

A noticeable point was the tendency for the development of acidity followed by a reversion to alkalinity among a majority of the strains. This reversal was much more common in mannite, saccharose, and salicin than in dextrose or lactose. The medium was a semi-solid agar with 1% sugar and 0.1% Andrade indicator. A few strains were tested in broth.

Typical B. coli-communis reactions (i.e., development of gas and permanent acidity on dextrose, lactose, mannite and salicin with no reaction on saccharose) were given by 3 strains only, 2 isolated from normal persons (Ban. and R.) and one from a specimen of W. recorded in another paper. Only 1 of the 2 normal strains was motile.

Typical B. coli-communior reactions, i.e., development of gas and permanent acidity on dextrose, lactose, mannite and saccharose, with no reaction on salicin were obtained from the specimens of one person (W.).

Gram-negative bacilli not fermenting or only faintly fermenting lactose were isolated from the specimens of several persons.

A strain giving the characteristic B. proteus reactions—no fermentation of lactose, with fermentation of dextrose and saccharose and liquefaction of gelatin—was isolated from Ck.

Several strains were isolated from J. which grew in a thin spreading moist film on agar slants and which liquefied gelatin, but they did not ferment saccharose and they gave a positive or questionable Voges-Proskauer reaction.

In summarizing the results, it may be concluded that gram-negative bacilli of the colon group, as well as nonlactose or faintly lactose fermenting organisms, are common at the levels studied. They were found in the specimens of both normal and diseased persons. The statements

in much of the literature about the presence in the small intestine of B. coli are too vague for us to compare them with our results.

It may be pointed out that especially large numbers and varieties of gram-negative bacilli were isolated from J., the patient with pyelonephritis and cystitis. The conditions for their multiplication seem to have been particularly favorable, and it is not impossible that infection of the kidneys may have derived from the intestines.

GRAM-POSITIVE STREPTOCOCCI

As was stated in an earlier paragraph, gram-positive streptococci or diplococci were found in about 80% of our specimens.

Thiercelin [53] described an organism called by him Enterococcus, which he isolated regularly from the feces and also sometimes from the mouth, nasopharynx, pus of cerebrospinal meningitis, etc., and which he distinguished from the streptococcus chiefly because its mode of division appeared to be much more complicated. He noted its extreme polymorphism.

Hirsch [54] and Libman [55] earlier described a streptococcus isolated from a case of infantile enteritis, corresponding in many of its characteristics to the enterococcus of Thiercelin. Kruse [56] expressed the opinon that streptococcus lacticus found in sour milk was identical with the streptococcus of Thiercelin. Andrewes and Horder,[57] Holman,[58] and Smith and Brown [59] have all classified streptococci derived from fecal sources, without apparently making a distinction between them and Enterococcus.

Our observations on intestinal cocci corresponded to the description given by Hirsch and in many respects to that given by Thiercelin. The cocci found were distinctly pleomorphic. Sometimes they showed a strong tendency to the diplococcoid or tetrad formation; at other times, they appeared in short chains. No constant morphologic difference was observed between the cocci-producing green on blood-veal agar and those that did not change the mediums. Since forms similar to those we found in the intestine are found in the throat, it seems more logical to record all our gram-positive cocci regularly forming short or long chains in broth mediums according to the classifications of Smith and

[53] Compt. rend. Soc. de biol., 1902, 54, p. 1082; 1903, 55, pp. 24, 686, 701, 750 and 798
[54] Centralbl. f. Bakteriol, I. O., 1897, 32, p. 369.
[55] Ibid., 1897, 22, p. 376.
[56] Ibid., 1903, 34, p. 737.
[57] Lancet, 1906, 2, pp. 708, 775 and 852.
[58] Jour. Med. Res., 1916, 34, p. 377.
[59] Ibid., 1914-15, 26, p. 455.

Brown,[59] as alpha, beta or gamma streptococci; and according to that of Holman [58] based on sugar reactions. It seems desirable to drop entirely the term Enterococcus in the literature dealing with the intestinal flora. Dible,[60] however, who asserts that correlation of characters should be the basis of classifying streptococci, suggests that streptococci which show heat resistance, short chain formation and fermentation of mannite, three characters having a high grade of correlation, should be grouped under the term "enterococci."

As is indicated in table 2, we isolated many strains from pathologic specimens that produced a green discoloration on blood-veal agar. In most of them, this metabolic activity was far less pronounced than in the majority of nasopharyngeal strains derived from persons with influenza or common colds, in the testing of which we had on a previous occasion assisted.[61] Unlike the streptococci of nasopharyngeal origin with which we had worked, most of the intestinal strains did not discolor, or only faintly discolored "chocolate" agar. Many of these intestinal green-producing strains seem to occupy a middle ground between the gamma and the alpha types of nasopharyngeal origin.

Strains from the same person often differed greatly in their ability to grow on artificial mediums. We found that the addition of sugar to the mediums enormously increased the luxuriance of most strains. N. and J. Mutch [23] have emphasized the glycophilic and acid resisting nature of intestinal streptococci. They reported only work on streptococcus longus. While admitting that length of chain cannot be correlated with pathogenicity, they restricted their observations to cocci having more than fifteen individuals to a chain, and usually distingushing themselves from the cocci in normal individuals by the scantiness of their growth on artificial mediums. Glycophilia they regarded as a striking characteristic of this group. It is found in some throat strains, but is absent in streptococci from other sources.

Most of the streptococci isolated by us were of the short chain variety, according to N. and J. Mutch; i. e., less than 15 units to a chain. We occasionally came on excessively long chains in the stained films of some of our liquid mediums. As we did not, however, succeed in isolating them, we could not determine whether they were the same streptococci found on other mediums, forming chains under special conditions, as Thiercelin said was done by his enterococci, or whether

[60] Brit. Med. Jour., 1921, 2, p. 789.

[61] Williams, A.; Unneberg, A.; Goldman, A., and Hussey, H.: Jour. Immunol., 1921, 6, p. 53.

they were distinct strains. One of the few moderately long chain streptococci which we isolated directly from our pour plates was obtained from the specimen of B. giving a previous history of acute endocarditis. It was fairly delicate, not aciduric, since it did not grow on the acid beef liver plate, nor markedly glycophilic.

N. and J. Mutch [23] also found that when intestinal streptococci, as opposed to those from other sources, were incubated with B. coli for several days in dextrose broth, the streptococci entirely suppressed B. coli. We found this, as well, to be the case in our anaerobic cooked meat broth tubes, to which no sugar had been added. The streptococci repeatedly overgrew the gram-negative bacilli. From the specimen of J., for instance, although the plates showed an overwhelming predominance of gram-negative bacilli, the cooked meat tubes developed streptococci almost exclusively.

Another observation made by these authors corresponds to our findings, although in our case results similar to theirs were noted with short chain streptococci. They say:

> It is noteworthy in this connection that in alimentary foci containing high proportions of Streptococcus longus, large numbers of stout, spore bearing, gram-positive anaerobic bacilli, morphologically resembling B. perfringens, are frequently found, and that the relative prominence of each organism often varies in a parallel manner when a series of observations are made on the same patient, under various conditions. The anaerobe often grows freely in symbiosis with Streptococcus longus even in aerobic meat broth cultures.

The symbiosis, however, we found did not persist beyond one generation in liquid aerobic medium. An example confirming the quoted statement is found in the two specimens of Sh. In the first of these, an anaerobe was isolated in almost pure culture from the highest dilution of cooked meat broth tube, streptococci being present in only small numbers in the lower dilutions, while in the second specimen, streptococci predominated throughout the cooked meat broth tubes.

Most of the sugar classification on streptococci was done on strains isolated from specimens of W., which will be recorded in another paper.

STAPHYLOCOCCI

While a few Staphylococcus albus colonies were often found on surface plates, it was suspected that they might be due to contamination. As staphylococci did not seem to be present in quantities in any of the specimens, no particular observations were made or recorded on this group.

SARCINAE

Although cocci in tetrads and irregular pacquets were often found on the stained films of the liquid mediums, bacteria that might be grouped among the sarcinae were isolated from the specimens of but a single individual. This was a most interesting case. It was taken with a tube length of 65″ and yielded only cocci on all mediums. Morphologically, all the cocci were indistinguishable, except that some forms were larger than others, yet culturally they divided themselves into 5 distinct groups. Morphologically they appeared as cocci found chiefly in tetrad formation, while some were in pacquets, or formed loose chains. The separate units were split in the center, forming two

TABLE 5

Reactions of Sarcina-Like Cocci Isolated From the Intestinal Specimen of H.

Morphology	Cultural Characteristics	Sugar Reactions				
		Dextrose	Lactose	Mannite	Saccharose	Salicin
Gram-positive cocci chiefly in tetrads, also in pacquets, irregular clumps and short chains in broth; each unit a diplococcus surrounded by a transparent capsule; in some fishings the tetrad formation more pronounced	B_1 Blood veal agar; simple colony with wide zone of hemolysis; a few corpuscles over colony; alpha prime type	+++	++	—	+	+
	S_1 Blood veal agar; typical alpha colony; blood drop agar slant, delicate growth	+++	+++	—	—	+++
	S_2 Agar slant, heavy lemon yellow growth	+++	—	—	—	—
	D_2 Blood veal agar: pin point colonies with small zones of clearing; alpha type	+++	+++	—	—	—
	D_3 Blood veal agar: no zones of clearing; agar slant, heavy white growth	+++	+++	—	+++	+++

flattened individuals surrounded by a clear capsule. One strain gave a heavy lemon colored growth on an agar slant, corresponding in cultural characteristic to the description of Sarcina lutea or flava of Lehmann and Neuman. Another strain gave a heavy white growth on an agar slant and corresponded in cultural characteristics to the description of Sarcina alba of Lehmann and Neuman. Three strains both in colony type and growth on agar resembled streptococci of the alpha type, although each of these also was distinct from the others in its sugar reactions. None of them, however, could be distinguished morphologically. All showed the tetrad arrangement, and the occasional chains in broth. Table 5 gives the details of the observations. We were

unable to obtain a second specimen from this person in order to determine whether the peculiar flora was characteristic or fortuitous.

In conclusion, we wish to stress the fact that experience with the class of cocci confirms the observations we made on the colon group, namely, that what superficially appears to be a pure culture of one organism, will on further analysis split up into several culturally distinct species or varieties. Predominance of similar gram-negative bacilli or of morphologically identical streptococci does not imply predominance of a single variety. We found in our study of alpha streptococci [62] from the throats of patients having influenzas or colds that the same person might harbor at least 2 or 3 strains distinct in their immunologic and fermentative properties. At the levels of the intestine studied by us similar subgroupings based on slight metabolic differences may be found.

NONSPORE-BEARING GRAM-POSITIVE BACILLI OF THE ACIDURIC GROUP

Bacteria of this general class were isolated, or their presence established by special culture methods, in about 49% of the pathologic specimens. In several of the persons from whom we obtained more than one specimen (V., J., W.) they were found in two or more samples of the intestinal contents,—an indication that they may constitute a constant factor of the bacterial flora at the levels studied. They were found in one only of our normal specimens. In all but 3 of the specimens they accompanied a very high bacterial count. This may be due to the fact that they constituted an insignificant proportion of the total flora, and therefore did not develop where the entire number of organisms was small. Furthermore, their symbiotic character, evidenced by their proportionally greater numbers on a crowded than on a thinly populated plate, may account for their apparent absence in bacterially poor specimens. In only a few cases was this group at all prominent on the acid beef-liver plates. Usually examples were isolated from the milk or the dextrose broth fermentation tubes, where they might in time overgrow all other species, or they were found in pure culture, or in symbiosis with streptococci, in the N/10 dextrose acetic acid tubes.

Torrey and Rahe [63] described a new member of the aciduric group, B. acidophilus-aerogenes, which produces gas in acetic acid broth. We found that some of our strains, when grown in 2% sugar under a petrolatum plug, developed a small amount of gas.

[62] Goldman, A.: Ibid., 1922, 7, p. 361.
[63] Jour. Infect. Dis., 1915, 17, p. 437.

SPORE-BEARING AEROBES

Little work was done on this group beyond establishing the presence or absence of such forms in the specimens.

Bacilli of the species B. mesentericus were isolated from three specimens, but were doubtless present in other specimens. Other organisms were tentatively classified as B. pumilus, B. megatherium, B. cereus and B. mycoides. Each of these were isolated a single time.

TABLE 6

APPROXIMATE PROPORTION OF ANAEROBES TO AEROBES

Specimen	Number of Organisms per C c. on Aerobic Plate Showing Highest Count	Probable Number of Anaerobes per C c.	Proportion of Anaerobes to Aerobes
V. II	12,000,000	>1,000,000	>1:12
Sb. I	320	100,000 to 1,000,000	>310:1* <3100:1
A.	280,000	1,000 to 10,000	>1:280 <1:28
W. III	295,000	>100,000	>1.3
W. VI	3,240,000	>100,000	>1:32
Meach.	1,600	10,000 to 100,000	>6:1** <60:1

* In this case the spore-bearing aerobes developed in the cooked meat tubes better than on the aerobic plates. They were present in numbers of 10,000 to 100,000 per c c. so that the actual proportion of anaerobes to aerobes was about 10:1.

** In this case, streptococci were also present in the last cooked meat tube which showed the presence of anaerobes, so that the number of aerobes and anaerobes was really about the same.

ANAEROBES

The study of anaerobes requires a specialized technic and is most time consuming. We desired not merely to isolate these organisms, if present, but above all to establish some rough estimate of their numbers, as this has not been successfully done for the nonfecal intestinal contents. The mere differences between the count on an aerobic and an anaerobic plate gives misleading estimates because B. acidophilus and many strains of freshly isolated streptococci are favored by anaerobic conditions. To our first 8 specimens we did not apply the method of inoculating cooked meat broth tubes with increasingly dilute suspensions of the original material. Out of the remaining 9 specimens revealing the undoubted presence of an anaerobe we were 6 times able to set a lower limit or to establish an upper limit for the number of these bacteria. These results are given in table 6.

It is interesting that twice (Sh. I, Meach.) large numbers of anaerobes should have been associated with facultative aerobes that hardly developed on the plates, but were favored by the cooked meat mediums. In three cases (W. III, W. VI and Meach.), the anaerobes were associated with a preponderance of streptococci among aerobes. Once (Sh.) they were found together with large numbers of spore-bearing aerobes, and twice (A. and V. II) with a preponderance of gram-negative bacilli, although in the specimens of V. II about 36% of alpha streptococci were also found on the blood veal plate.

We did a considerable amount of detailed study on the anaerobes, but as Hall,[64] Kahn[65] and Heller[66] have emphasized, nothing but repeated isolation, preferably, according to Kahn, by the single cell method, will insure purity. There is particular danger of contamination with B. sporogenes. This organism we isolated four times. In no case was there evidence of B. sporogenes being present in large numbers. Twice it was isolated from the undiluted heated material. We are inclined to regard its presence in the intestine as fortuitous—a few spores may be introduced with the food—rather than to look on it as an intestinal parasite. When anaerobes were isolated from high dilutions of the original material, they were always less strongly proteolytic than B. sporogenes.

B. welchii was probably isolated from the specimens of J. I, W. III and W. VI, although some of our cultures appear to have been contaminated with a mildly proteolytic organism, as there were traces of liquefaction in the inspissated serum.

In conclusion, we may state that anaerobes are likely to be present in large numbers when the number of aerobes is also great. As they are found in specimens in which other groups such as the aciduric bacilli may also exist in considerable numbers, there seems to be no reason to ascribe to them, without further evidence, any particular pathologic significance, unless their absence in any great concentration in the upper small intestine of normal persons should be confirmed in more cases than we have been able to handle. Possibly when they are found in large numbers in a specimen with a low aerobic plate count but with a high count in the dilution tubes of cooked meat mediums, as was the case in samples from Meach. and especially from Sh., they may indicate a more marked putrefactive activity than is usual at these

[64] Ibid., 1922, 30, p. 445.
[65] Jour. Med. Res., 1922, 43, p. 155.
[66] Jour. Bacteriol., 1921, 6, p. 445.

intestinal levels. Their presence, when the diet is poor in meat, may also be an unfavorable symptom.

GRAM STAIN

It was not always possible to make a differential count, as sometimes the bacteria in a specimen were too scarce to find under the microscope. At times, probably because of the presence of bile salts, we encountered difficulties in getting the material to adhere to the slide.

The number of bacteria on the slide seems to have little relation to the number of viable organisms. In one case (P.) in which the specimen yielded a very low colony count, the slide was covered with bacteria. In this case the olive, according to the roentgen-ray report, was in the duodenum, and it is possible that many dead bacteria from the stomach contents were still visible.

In most of the samples, gram-positive bacteria, frequently gram-positive cocci, were predominant. We did not find a close relationship between the microscopic and cultural findings. The specimen of Jol., in which we found a great preponderance of gram-negative bacilli, had a differential count, showing 70% gram-positive diplococci.

The two specimens from a normal person, Fr., taken about 10 inches apart, and both on cultivation showing a preponderance of gram-negative bacilli, gave the following differential counts:

	Gram-positive Bacilli	Gram-positive Cocci	Gram-negative Bacilli	Gram-negative Cocci
50″	16	80	4	0
40″	42	25	30	3

One must assume that clumps of bacteria, many of them nonviable, are unevenly distributed through the chyme, and appear in different parts of the microscopic field.

I believe that the microscopic picture, especially shortly after eating, is a poor indication of the viable bacterial flora.

DISCUSSION

In the introductory remarks it is stated that a study of the bacterial flora of the upper small intestine in its relation to disease might prove of importance if it were possible to set broad limits to the number of viable organisms at any level, consistent with a healthy condition of the intestine, and to determine whether the presence of certain bacterial

species, perhaps normally to be found at other levels, seemed to be associated with pathologic symptoms. It has not so much been our object definitely to establish these points, as to discover whether they are susceptible to investigation that may in the future yield significant information, and along what lines research on this theme may prove most valuable.

Our study of 4 "normal" persons revealed the fact that bacterial numbers in the jejunal region may vary within exceedingly wide limits, and yet be consistent with an apparently healthy condition of the gastro-intestinal tract. On the other hand, we found that when the diseases of our patients were classified according to the number of bacteria found in 1 c c. of their intestinal contents, those patients whose symptoms were apparently unrelated to gastro-intestinal conditions rather consistently revealed a moderate bacterial count. On the other hand, persons suffering with diseases directly accompanied by serious gastro-intestinal symptoms (pernicious anemia) or with diseases repeatedly associated in medical literature with possible foci of infection, primary or secondary, along the alimentary tract (arthritis deformans, articular rheumatism), or with a bacterial infection possibly emanating from the intestinal wall (pyelonephritis), these persons were distinguished by the much higher bacterial count obtained from their intestinal specimens. One or two persons, whose diseases did not seem in any way traceable to an intestinal origin, also fell into the high count group. Repeated tests on several of the cases, yielding results that corresponded well, seem to indicate that these groupings may not have been entirely due to chance.

We inclined to the opinion that the conflicting results derived from our normal and diseased cases might find their explanation in the different dietary regime under which the two groups were living. We noted that several of the medical cases suffered from poor appetite. They were confined to bed and were restricted for the most part to the "soft special" diet. The poor appetite of the patients while in bed was probably an important factor in keeping down bacterial numbers when other causes did not increase them. It may, therefore, be that high bacterial numbers in the presence of a light diet point to some condition such as peristaltic stagnation, impaired activity of the intestinal enzymes, or delayed absorption, that appears to offer a suitable environment for the multiplication of bacteria either normally present or associated with a focal area of infection.

The question of the presence of certain bacterial species we found must also be treated, as indeed all previous work on the relation of fecal bacteria to food would lead us to expect, with reference to the dietary background. In addition to the invasive intestinal bacteria known to cause diseases of definite pathology, such as those of the typhoid-paratyphoid group, other bacteria normally present in small or large numbers have been held responsible for disorders of which the etiology has been less successfully traced.

Thus the presence of large numbers of the colon group in the upper small intestine has been supposedly associated, especially in children, with dyspeptic manifestations, and in adults with biliary infections, hypo-acidity and pernicious anemia.

Herter [14] postulated 3 types of intestinal putrefaction: (1) the indolic, associated with the B. coli group; (2) the saccharobutyric, initiated especially by anaerobes; and (3) the mixed indolic and saccharobutyric type. Apparently he considered B. coli in the first case, and B. welchii in the second, as directly responsible for the symptoms associated with their presence, and his point of view led to the conception that the substitution of a more benign flora would also remove the source of the derangement. The more recent work on the feeding of B. acidophilus, following up the conception of Metchnikoff, has also assumed that whatever cause may have stimulated the development of an unfavorable flora, the suppression of this flora is the essential factor in clearing up conditions that are often only indirectly associated with gastro-intestinal symptoms.

Finally, the group of nonhemolytic streptococci are believed to form areas of focal infection in the intestines as well as in the tonsils and teeth, in a great variety of pathologic conditions.

One fact that struck us forcibly, and which I wish especially to emphasize, is the mixed bacterial character of most of the specimens, both normal and pathologic, even where one type of organism was strongly predominant. We found a certain number of specimens in which one species seemed almost or entirely to exclude other species, but for the most part the flora, like that of the feces, presented a varied picture, made up of bacteria that may be considered as having antagonistic metabolic requirements.

We found that the colon group is a normal inhabitant of the jejunal region. We isolated B. coli communis from the specimen of a normal person with a very low count, and we found a predominance of gram-

negative bacilli having many of the cultural characteristics of B. coli or of B. aerogenes in the contents of two other normal persons. In view of these findings, a great predominance of the B. coli group associated with a high count in a case of pernicious anemia (Jol.) can hardly be regarded as particularly significant. We noted, however, that while the gram-negative bacilli isolated at these levels corresponded in many respects to the descriptions of B. coli, they gave certain atypical sugar reactions; that is, they showed a tendency to revert to alkalinity after acid production. It may be that a classification of these bacteria as exhaustive as that which has been carried out on gram-negative bacilli originating in the feces and in water could distinguish between harmless and more pathogenic types. For instance, it is possible that the condition which promoted the multiplication of so many gelatin-liquefying gram-negative bacilli in the upper intestine of a person with pyelonephritis (J.) may also have lowered the resistance of the intestinal mucosa to bacterial penetration.

When we consider the question of putrefaction and its relation to the presence of anaerobes, we find the situation difficult to analyze. Macfayden, Sieber and Nencki [7] decided, from a chemical investigation of fistula material, that there was little putrefactive activity in the small intestine. Herter [14] held that native proteins can undergo bacterial decomposition in the intestine only through the action of anaerobes, and that the defensive action of the gastric juices and rapid passage of the chyme account for the freedom from anaerobes in the upper small intestine. Kendall [15] stated that the initial breakdown in putrefaction is brought about by obligate anaerobes, while the process is carried to its lowest terms by facultative anaerobes.

Anaerobic bacteria could not be isolated from our four specimens derived from normal persons. If present, they must have formed an insignificant percentage of the total flora. On the other hand, we found considerable proteolytic activity in the milk and cooked meat medium, with spores in the sediment of the sugar-free broth fermentation tubes. In our specimens derived from hospital patients, we many times found anaerobes, but putrefactive tendencies indicated by the presence of abundant spores in the sugar-free broth fermentation tubes and by peptonizing of milk were relatively less common than in normal specimens. We ascribed the evidences of proteolysis in the specimens from normal persons largely to spore-bearing aerobes, favored by a diet containing meat, while the insignificance of these organisms among

many of the specimens from our pathologic cases, perhaps attributable to their meat-free diet, would account for fewer spores in the sugar-free fermentation tubes and less frequent peptonizing of milk. We thought that where anaerobes were not present, the combined activity of the diluted digestive ferments and some types of cocci or gram-negative bacilli might account for partial digestion of the meat in the lowest dilution of the cooked meat tubes. It is of course possible that conditions in the small intestine, i. e., the presence of sugars, and rapid digestion of proteins by the intestinal enzymes, normally discourage putrefactive activities, but it seems probable to us that a certain amount of bacterial protein decomposition is regularly taking place, and that only when the organisms producing such activity appear in excessive numbers, even in the presence of a low protein diet, as was for instance the case in the specimens of Sh., may the evidence of putrefactive activity in the culture medium assume diagnostic significance. It might indicate a condition of the intestinal mucosa favorable to the development of putrefactive organisms such as appears, for instance, to exist after a prolonged period of starvation, or it might point to a previous implantation of these bacteria too firm for a low protein diet easily to dislodge.

Streptococci or diplococci were present in all of our specimens from normal persons. Thus we cannot agree with N. and J. Mutch,[23] who thought that they were probably normally present in small numbers only in the small intestine. We did not, however, find the types producing a green discoloration on blood agar plates, although these are normally present in the feces. As in the case of the anaerobes, I believe that our data on healthy persons is too meager for us to draw any final conclusions. On the other hand, we found the green-producing type present in the greatest variety of pathologic conditions.

Regarding the question of a normal or obligate flora of the small intestine, we found that certain bacterial types were common while others were always absent.

In analyzing the normal flora of the upper respiratory tract, Bloomfield [67] has shown that certain groups of organisms are usually present in most persons, that other organisms are regularly absent, but that certain other groups, hemolytic streptococci, pneumococci, etc., may temporarily dislodge the usual bacteria and become for the time being the new normal flora of a person without necessarily initiating disease.

[67] Am. Jour. Med. Sc., 1922, 164, p. 854; Bull. Johns Hopkins Hosp., 1921, 32, p. 33.

I believe that conditions in the small intestine may be somewhat analogous to those in the upper respiratory tract, but that possibly persons vary more, due partly to habits of diet, in the range of organisms which they normally harbor. While we noted the persistence of the colon or the streptococcal or the acidophilus group in several specimens from the same person, nevertheless the prominence of different types varied considerably from time to time. It is impossible, of course, to judge to what extent the intestinal flora is independent of the bacterial contents of the ingesta. I incline, however, to the view that the small intestine has a native flora, susceptible of change, which is independent of this factor. The flora may be in part derived from the normal mouth bacteria. Nonhemolytic streptococci are a prominent feature of both centers. The whole question is susceptible of investigation but has not been studied by us, except in one case, to be recorded in another paper.

What is the value of a bacterial study of the contents of the small intestine, and may bacterial numbers or bacterial species throw any light on the etiology of obscure pathologic complexes? The bacterial picture, regarded independently of other considerations, such, for instance, as the nature and quantity of the diet, will not reveal very much. A single specimen is inadequate. The same person should probably be studied after a fast as well as after regular eating. For routine purposes, except for investigating a bacterial infection of the bile, the procedure of swallowing and retaining the intestinal tube seems to be far too difficult and too trying to the endurance of the patient to be recommended. Nevertheless, it seems possible that in studying certain diseases, the bacterial findings in the intestinal contents, taken together with chemical data and with observations on the tonsils, the stomach, the urine, the blood, etc., might prove of distinct value. It might, for instance, be possible to determine by serologic methods whether gram-negative bacilli in the intestine are the same as those found in the ureter in pyelonephritis, and thus help to establish the original source of bacterial invasion.

The problem of focal infection is of immense importance in the arthritic and rheumatoid conditions, and the most refined technic should be applied in determining whether bacteria which are disseminated from foci in the upper respiratory tract proliferate in the intestine.

In any case, it seems desirable to study a greater number of normal persons and to extend, if possible, the investigations into the region of the colon.

CONCLUSIONS

The following conclusions are arrived at from a study of the contents of the human small intestine, chiefly at the jejunal level:

The number of viable bacteria in the contents of a number of normal persons and hospital patients varied within wide limits.

The number of viable bacteria in the contents of diseased persons confined to a light meat-free diet usually appeared to be considerably higher in patients with gastro-intestinal disturbances or with diseases associated with metabolic disturbances or with focal infections of possible gastro-intestinal origin than in patients with diseases apparently unconnected with the alimentary tract.

The flora, in normal and pathologic cases, was usually of mixed bacterial character, forms, with widely varying metabolic requirements, multiplying in the same specimen.

Even when the contents were entirely dominated by one bacterial species, further analysis into cultural and biochemical properties revealed the presence of two or more varieties.

Several specimens from the same person taken under similar conditions usually showed approximate correspondence in bacterial numbers and in the predominant species.

Gram-negative bacilli of the colon group, gamma type streptococci, and gram-positive bacilli of the acidophilus group appeared to be a normal feature at the levels studied.

Spore-bearing aerobes, accompanied by proteolytic activity, appeared to be a normal feature of the intestinal contents.

Anaerobes were absent in four normal specimens but were found in many specimens from pathologic subjects, but not necessarily in all specimens from the same person. Large numbers of spore-bearing aerobes were not as common as in specimens from normal persons.

The presence in considerable numbers of spore-bearing aerobes we attributed in our normal subjects to the presence of meat in the diet.

STUDIES IN INTESTINAL BACTERIOLOGY

II. THE EFFECT OF SPECIAL FEEDING ON THE INTESTINAL FLORA

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In the first paper in this series, I discussed the bacterial flora of the small intestine of 4 normal persons and of 20 hospital patients.

One of the female patients W. (arthritis deformans) was particularly ready to take the intestinal tube, and was most conscientious in following directions. She was kept at the hospital for several months, at the request of Dr. Coleman, receiving treatments in the form of bakings, massage and vaccine injection for the arthritic condition.

Altogether 20 intestinal specimens were obtained from her. The methods were the same as those described in the earlier paper. In the work with B. acidophilus we attempted to make use of whey agar plates as recommended by Rettger and Cheplin[1] with but little success.

During much of the time she was receiving B. acidophilus culture (Arlington Chemical Co.), usually 6 teaspoonfuls a day. Later we isolated the strain from one of the bottles, and prepared with it cultures in dextrose broth. The strain isolated corresponded morphologically and culturally to descriptions of B. acidophilus, except for the fact that it did not ferment maltose. We do not know whether this strain never fermented maltose, and therefore did not correspond to the usual definition of B. acidophilus, except for the fact that it did not ferment maltose. We do not know whether this strain never fermented maltose, and therefore did not correspond to the usual definition of B. acidophilus, or whether it had lost this property.

The number of viable bacteria in the broth cultures, which were tested from time to time, varied considerably. The strain was extremely capricious. A fresh 18-hour culture had at least 200,000,000 viable bacteria per c c., and sometimes several billion.

Table 1 is divided into two parts. In the first part are given the results while the patient was at the hospital on regular hospital diet receiving treatment and showing considerable improvement in her general condition. During most of the time she was getting 6 teaspoonfuls a day either of the commercial preparation called Bacid, or of our laboratory preparation. There is reason to believe that the culture which she was taking from June 20 to July 20, and which was of our own preparation, was largely nonviable, as it was probably kept too long.

All recent experiments agree that such small doses of B. acidophilus, especially if they are not reenforced with lactose feedings, are entirely ineffectual in changing the flora, and we may, therefore, look on this first set of records up to July 6 as observations under normal dietary conditions.

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[1] Intestinal Flora, 1921.

TABLE 1

STUDY OF INTESTINAL CONTENTS OF W. OVER A PERIOD OF FOUR MONTHS

SUMMARY OF RESULTS UNDER NORMAL CONDITIONS AND AFTER SPECIAL FEEDING

Date	Nature of Specimen	Feeding	Number of Bacteria per C c. of* Intestinal Contents or of Fecal Suspensions on			Predominant Bacteria on Aerobic Pour Plates	B. acidophilus Group	Approximate Percentage# of B. acidophilus Group of Total Aerobic Count
			Endo Plates	Blood Veal Plates	Acid Beef Liver Plates			
5/ 3	Intestinal, approximately 70 to 80″	.....	10,200	1,200,000	3,240,000	Streptococci	+	< 1
5/19	Intestinal,† 70″	5/4–5/21: 6 teaspoonfuls of Arleo B. acidophilus culture daily	3,000,000	45,000,000	66,000,000	Colon group	+	< 1
6/ 2	Intestinal, approximately 50 to 60″	.....	3,000	4,000	4,000	Streptococci	—	< 1
6/ 7	Intestinal, approximately 50 to 60″	6/3–6/13; 6 teaspoonfuls of Arleo B. acidophilus culture daily	< 20	7,200	2,400	Diplococci	+	< 1
7/ 6	Intestinal, approximately 45 to 50″	6/28–7/7: 150 gm. lactose daily....	550,000	1,062,000 (80% streptococci)	1,457,000	Streptococci	+	< 1
7/14	Rectal swabbing	7/7–7/13: 100 gm. lactose daily....	1,500,000	2,000,000 (37% streptococci)	5,300,000 (22% B. acidophilus)	Colon group	++	20

SUMMARY OF INTENSIVE FEEDING ERPERIMENTS WITH B. ACIDOPHILUS AND LACTOSE

8/ 2	Intestinal, 90″	7/19–7/28: 6 teaspoonfuls laboratory culture of B. acidophilus freshly grown every 3–4 days	600,000	6,000,000	9,400,000	98% streptococci	++	> 1
8/ 2	Rectal swabbing	7/28 and 7/29: 100 gm. lactose daily; 7/30 and 7/31: 50 gm. lactose daily; 8/1; no lactose	100,000	1,600,000	400,000 (30% B. acidophilus)	Colon group	++	5–10
8/ 5	Intestinal, 100″	8/2: 30 c c. laboratory culture of B. acidophilus strain fed through 90″ tube; 8/3 and 8/4; no feedings	820,000	1,450,000	1,370,000	Streptococci	+	< 1
8/ 5	Rectal swabbing	.....	1,600,000	1,700,000	1,300,000	Colon group	++	4–5
8/11	Intestinal, 100″† Some coiling in stomach, aproximate position doubtful	8/5–8/7: no feedings; 8/8: 75 c c. of laboratory culture of B. acidophilus strain fed through 100″ of tube; 8/9, 8/10: no feedings	3,050,000	5,520,000	7,310,000	Streptococci	++	> 1.5
8/15	Intestinal, 80″	8/11: 75 c c. of laboratory culture of B. acidophilus fed through 100″ # of tube plus 100 gm. lactose; 8/12, 8/13, 8/14: 100 gm. lactose daily	< 20	13,400	13,600 (yeast)	Streptococci and sporing gram-positive bacilli	—	< 1
8/15	Rectal swabbing	.....	109,200	108,400	268,000	Colon group	—	< 1
9/14	Intestinal, 65 to 70″ †	8/22–9/15: 250 c c. daily of laboratory culture of B. acidophilus strain plus 100 gm. lactose daily	29,800,000	16,800,000	14,700,000	Streptococci and colon group	++	1.5
9/14	Fecal swabbing	.....	85,000	125,000	148,000	Colon group	+	< 1

++, present in large numbers or amounts; +, present; —, not present as far as observed.

* The fecal suspensions from rectal swabbings probably represented between 2–10 milligrams of feces per c c.

† The high counts on these intestinal specimens indicate that a reversed peristalsis may have taken place.

\# The approximate percentage of B. acidophilus was estimated by noting the percentage of colonies present on the acid beef-liver plates and by noting the highest dilution of the N/10 acetic broth tubes in which morphologically characteristic bacilli developed.

The first 4 specimens, taken over a period of 1 month, show greater variation in bacterial numbers than the earlier specimens. A count of 66,000,000 was far in excess of anything found in the previous specimens of this patient. No reason was found to account for the sudden increase in numbers or the predominance of gram-negative bacilli, which gave typical B. coli-communis sugar reactions, but it is possible that we here have an example of reversed peristalsis such as later occasionally was found to take place. The next 2 specimens showed an unusually low count. Another sample not included in the table, taken June 14, with an open olive, showed a count of less than 20,000 bacteria per c c.; thus over a period of about 2 weeks, 3 successive specimens gave evidence of a decided reduction in bacterial numbers. Whether this reduction was related to the patient's generally improved condition or is attributable to the somewhat higher level from which the specimens came, we are not in a position to state. Some coiling of the tube was reported, and it was difficult to estimate the depth of the olive.

On June 28 she started taking lactose, 150 gm. per day until July 7, followed by 100 gm. per day from July 7 to 13. On July 3 a mild diarrhea set in. A specimen taken on July 6, shows a rise in bacterial numbers to over 1,000,000 per c c.

The last specimen is a rectal swabbing taken at the close of the period of lactose feeding. A suspension in broth or salt solution was made from all swabbings. The amount of material was not weighed, as comparative rather than absolute numbers were desired. Counts on the different plates poured from the same rectal or fecal specimen give indications of the proportions of different bacterial groups. One c c. of suspension represents approximately between 2 and 10 mg. of feces.

A noteworthy point is the fact that all intestinal specimens, except the one with an excessively high count, show a predominance of streptococci. Even the rectal swabbing shows a fairly high percentage (37%) of green-producing streptococci.

We may note another related fact, that in the case of all the high count specimens the plates poured with acid beef-liver medium are by far the most crowded, even in the one with a predominance of gram-negative bacilli. This indicates that the flora of this patient was glycophilic and decidedly aciduric. The feeding of lactose encouraged these aciduric and glycophilic organisms both in the small and large intestine.

On investigating the proportion of B. acidophilus, one finds that, as might be expected with such an aciduric flora, this organism was usually present although in small numbers. It often overgrew all other organisms in the milk tube, and it was frequently isolated from the N/10 acetic acid broth. The approximate percentage of B. acidophilus-like bacilli of the total aerobic plate count has been put down as less than 1% whenever there were too few present to make an estimate, although in many cases the proportion is probably much under 1%.

Since the members of this group were normally present, we should expect the lactose to stimulate their development. From the intestinal specimen of July 6, we now find a few characteristic colonies appearing on the acid beef-liver plates, but on the whole the sugar is much more strongly favorable to the streptococci. The most crowded, however, of the acid beef-liver plates, poured from the rectal swabbing, showed 22% of B. acidophilus colonies. The acid beef-liver plates poured from earlier rectal specimens had no colonies of this type. We may say, at this point, that our search for B. acidophilus was

thorough. We looked not only for the fluffy, but also for the smooth edged, type of colony; and we made many isolations and stained films from colonies whenever we were in doubt.

The second section of table 1 is given over to the record of an experiment in feeding a viable B. acidophilus culture through the tube, and then for two weeks administering large quantities per os, together with lactose. Our object in the first case was to avoid the action of the gastric secretion. We intended to give the patient a certain length of tube to swallow, to take a specimen, and then before removing the tube, to inject some culture into the intestine. The patient was then to have a day of rest, and on the third day we were again to give her the tube to swallow with the purpose of having the olive reach a point below the level attained on the previous occasion. The object was to ascertain, through the intestinal specimen, and through a rectal swabbing, whether B. acidophilus had established itself below the point of injection. We then intended to follow the same method of procedure, administering culture and lactose simultaneously, the lactose, however, being taken by the mouth.

The chief difficulty was in controlling and confirming the point reached by the tube. According to the roentgen-ray reports, there seems to have been some coiling in the stomach on several occasions. Nevertheless had B. acidophilus become established, we might expect it to spread over a considerable region.

Besides pouring the acid beef-liver plates with 3 dilutions we also made dilutions of the specimens in a series of N/10 acetic dextrose broth, using from 0.5 c c. of 1 : 10 to 0.1 c c. of 1 : 10,000 or 0.1 c c. of 1 : 100,000 dilution. Sometimes these tubes gave evidence of a growth which on the stained film morphologically resembled B. acidophilus, when no typical colonies could be found on the acid beef-liver plate. Often the bacilli in the N/10 acetic broth were nonviable—development was apparently initiated and then inhibited.

In obtaining the percentage of B. acidophilus, we made use both of the acid beef-liver plates and of the N/10 acetic broth tubes. If morphologically characteristic organisms were found on the stained film from a N/10 acetic tube made up with 0.1 c c. of a 1 : 1,000 dilution of the original material, while none was present in a tube containing 0.1 c c. of a 1 : 10,000 dilution, we considered that between 10,000 and 100,000 B. acidophili were present per c c. We then compared this number with the highest count on a plate having no B. acidophilus colonies. While it might be objected that many bacteria of different species which are too feeble to grow on a plate might develop in a liquid medium, and that therefore we were estimating our percentage of B. acidophilus too high, it is also true that N/10 acetic broth even with 2% dextrose is a severe medium, and that therefore a tube must be fairly heavily inoculated for growth to take place.

The two specimens, intestinal and rectal, taken on August 2, followed a week of B. acidophilus feeding (6 teaspoonfuls a day) and 3 days of lactose feeding. In the intestinal specimen over 1%, and in the rectal specimen from 5 to 10%, of B. acidophilus was present. After stopping the lactose, and feeding 30 c c. of culture directly through the tube, there was an apparent drop of B. acidophilus content both in the small intestine and in the feces. After feeding 75 c c. of culture, there was apparently a slight rise of B. acidophilus in the intestinal content, but a further drop in the rectal content. August 15 another 75 c c. of culture was injected through the tube, and on this and the following 3 days 100 gm. of lactose was taken. Neither the intestinal nor rectal specimens showed any B. acidophilus. Moreover, in none of the specimens was there a reduction of the amount of gas in the dextrose broth fermentation tubes. From

this experiment, we conclude that a single injection of a concentrated B. acidophilus culture directly into the small intestinal tract is of no value in establishing the organism. The results might be different if the tube were retained long enough for several injections to be made. We wish, however, to call attention to the presence of free spores in the sediment of the dextrose fermentation tube (the record of observations on this medium is not included in the table), and to the predominance of yeast on the acid beef-liver plates of the intestinal specimen of August 15. Kendall[2] observed that when animals are fed for some time on carbohydrates, gram-positive spore-bearing bacilli which slowly liquefy gelatin appear as a kind of intermediate adaptive flora. The spores in the sugar tube and the yeast seem to us to represent a type of intermediate flora appearing in response to a few days of lactose feeding.

Finally, we fed our patient for 2 weeks daily with 250 c c. of a laboratory culture in dextrose broth of B. acidophilus, together with 100 gm. of lactose. We then had every reason to expect an implantation. After this period the intestinal contents showed between 1 and 10% of B. acidophilus-like bacilli in the N/10 acetic broth, and no typical colonies on the acid beef-liver plates. We again note the great increase in total numbers observed in a single previous specimen. From a fecal swab we made an estimate of less than 1%. This gave lower results than had been given by a previous feeding with lactose alone. A fecal swabbing taken 4 days after the feedings were stopped showed about 5% of B. acidophilus colonies.

The question naturally arises whether the strain used by us, derived from a commercial bottle, was adapted to implantation. We stated at the beginning that this strain had lost, if it ever had possessed, the faculty of fermenting maltose, which is supposed to be characteristic of B. acidophilus. In this connection we wish to state that every strain resembling B. acidophilus isolated either from the acid beef-liver plates, milk or acetic broth tubes, and tried on maltose, invariably fermented this sugar, some strains more slowly than others. From this result we must conclude that there never was any implantation of the strain administered, and that fluctuations in the percentage of B. acidophilus were entirely due to lactose feedings or to some other fortuitous factor. Work carried on in our laboratory by Miss Otto indicated that with several other strains also true implantation did not take place.

EXPERIMENT WITH STERILE FOOD

The patient left the hospital and returned to her home on Oct. 1, 1922. November 20, she came late in the evening to the Willard Parker Hospital, where she was under our direct observation. That night she took a cathartic. From the following morning she was served with food that was as nearly sterile as possible. She received sterile water, boiled milk, oranges, boiled eggs, and thoroughly toasted bread. Her dishes were kept apart and boiled. She washed her hands thoroughly and gargled with a disinfectant before eating. The only nonsterile food she received was 100 c c. of a laboratory culture of the same B. acidophilus strain which she had previously taken. On Nov. 22 the tube was passed, and on the morning of the 23rd a specimen was taken. During this entire period her food was controlled.

The roentgenogram giving the position of the tube, of which 75″ were swallowed, showed that no coils were present.

Great difficulty was encountered in aspirating any material. It was finally necessary to inject about 20 c.c. of salt solution, followed by some air, to expel

[2] Jour. Med. Res., 1910, 22, p. 153.

it from the tube, and then after waiting from 5 to 10 minutes, to aspirate again. By doing this twice, enough material for cultivation was obtained.

With the exception of 3 surface colonies of staphylococci regarded as contaminations, three plates, each of which contained 0.05 c c. of the original material, were entirely sterile. So also was the dextrose broth fermentation tube, and the N/20 acetic dextrose broth. In the sugar-free broth fermentation tube we found a small diplococcus. A similar organism and a few small gram-positive bacilli were found in the stained film from the lowest dilution tube of the cooked meat medium. In the milk, which slowly coagulated, we found a minute, long chain streptococcus, 15 to 18 faintly gram-positive individuals constituting a chain. On cultivation, this strain formed minute colonies of the gamma type on a blood veal plate.

A first glance at the result of this experiment inclines one to the opinion that the bacterial flora of the small intestine may be determined by the bacterial features of the ingesta. There are, however, several reasons why such a conclusion seems unwarranted.

First, we must remember that several months had passed since the patient was discharged from the hospital, and that her general condition was much improved over what it had been during the first months after entering the hospital. She had not been taking any lactose, which, as we have seen, had a stimulating effect on the normal intestinal flora. Some recent experiments, not recorded here, have given confirmatory results, that is, they indicate a great natural reduction in the numbers of the intestinal flora.

Another important point is that in our effort to give her sterile food, we simplified her diet greatly. Except for a little bread, the food was liquid or semisolid, and for the most part easily digestible. Probably a large part of it was absorbed in the upper levels of the small intestine, and what remained passed rapidly into the colon without offering a nidus for the breeding of bacteria, such as would be afforded by masses of undigested meat or of carbohydrates.

The fact that the only organism successfully recovered was a delicately growing streptococcus, a member of the group most characteristic of the intestinal contents of this patient, would seem to suggest that here we have a representative of what, for this person, constitutes a part of an obligate indigenous flora. In the later experiments, just referred to, it was again found that the only recoverable organisms in two apparently almost sterile specimens were streptococci, this time of the alpha type, developing best in the milk tube.

Finally, the fact that the large numbers of viable acidophilus bacilli ingested were not recovered denotes that they passed directly out of the small intestine, without even temporarily establishing themselves.

Thus, it would seem to us that while bacteria introduced with the food or saliva may temporarily multiply in the interior of undigested masses of food, the bacterial numbers will rather depend on the amount and the nature of the diet than on its microbal constituents, while the basic flora, like that of the upper respiratory passages, will maintain itself through considerable environmental variations. It seems possible, however, that the rapidity with which the bacteria are eliminated may depend on the substratum on which they are introduced. Those in a liquid medium might be rapidly disposed of through the action of peristalsis, while those embedded in a more solid substance might multiply, especially in the presence of sluggish peristalsis, until they temporarily overshadowed or at least modified the picture of the permanent flora.

A similar series of experiments, over a protracted period, should of course be carried out on one or more normal persons, in order to determine whether a certain bacterial type is always predominant in spite of a variety of environmental changes.

SUMMARY

The strain of B. acidophilus fed in large quantities to one patient could not be implanted in the intestine. Lactose feeding was more effective in increasing the number of B. acidophilus. It seemed most effective, however, in increasing the number of streptococci.

Large numbers of viable B. acidophilus introduced directly into the small intestine were rapidly eliminated.

After a diet sterile except for a broth culture of B. acidophilus the intestinal content at a depth of 75″ was almost sterile, streptococci being the only recoverable organisms in a few tubes of liquid medium.

It is suggested that streptococci may represent the indigenous bacterial flora of this person at the level tested.

STUDIES IN INTESTINAL BACTERIOLOGY

III. A STUDY OF NONHEMOLYTIC STREPTOCOCCI FROM HUMAN INTESTINAL TRACT

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Investigations on the antigenic relationships of the Streptococcus viridans group from human throats,[1, 2] have demonstrated its heterogeneous character. From the same person at a given time, two or more culturally similar but antigenically distinct strains may be isolated.

It seemed of interest, therefore, to examine the streptococci isolated from the intestines of one person at the same and at different times. Opportunity for such a study was afforded by material obtained through the Einhorn tube from patient W. in the manner described in the previous papers of this series.

The existence of similar immunologic reactions in two or more strains isolated at different times might throw some light on the question of a native or permanent flora in the small intestine. When the patient, as was the case with our subject, is suffering from arthritis deformans, often ascribed to focal streptococcal infection, the frequent reappearance of the same strain might indicate a possible relationship of the strain to the disease.

N. and J. Mutch [3] state that there is a great increase in the absolute and relative numbers, and in the varieties of intestinal nonhemolytic streptococci in certain conditions of disease. Holman [4] observes that focal areas of streptococcal infection often contain more than one type of streptococcus. There is also evidence that when green-producing streptococci appear in the throat associated with colds or influenza, a greater variety of strains is present than is found in normal throats.[2]

The varieties isolated ranged from strains growing luxuriantly to those growing sparsely on ordinary dextrose agar, although none seemed as delicate as two strains obtained from a nasopharyngeal

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[1] Krumwiede, C., and Valentine, E.: Jour. Infect. Dis., 1916, 19, p. 760.
[2] Goldman, A.: Jour. Immunol., 1922, 7, p. 361.
[3] New York Med. Jour., 1921, 113, p. 713.
[4] Jour. Med. Res., 1916, 34, p. 377.

swabbing of the same patient. Some were of the gamma type; others were of the alpha type and produced a vivid green zone, followed by marked clearing after standing at room temperature, but most strains occupied an intermediate position, only slightly discoloring the blood, and developing a small hemolytic zone. Isolations were made from different mediums as indicated in table 1, which gives immunologic relationships, and sugar reactions of a number of strains derived from 9 intestinal and 1 nasopharyngeal specimen.

If one first examines the sugar reactions, it is seen that the intestinal streptococci fall into several groups. As might be expected, Strep. fecalis was isolated on several different occasions. Strep. equinus reactions were given by all strains tried on sugars from examination 4 (strains 11-14). They were originally rather delicately growing organisms. Strep. mitis, which, according to Holman, is the most common of the nonhemolytic strains, was recovered from 4, possibly from 5, specimens. Occasionally a sugar reaction was so faint (±) that without repeating the test it would be difficult to classify the strain. Strep. ignavus was twice isolated. Strep. salivarius was isolated from one intestinal specimen only.[19] Particular reference is made to this as it was unique.

The lower dilution blood plates from the original specimen showed a mixed flora, but the highest dilution plate was exclusively seeded with colonies of streptococci, which were eventually found to constitute over 98% of the total flora. On further analysis, the streptococci from this sample fell into two groups: one, represented by strain 21, formed short chains in broth, and moderately green-producing colonies on blood agar; the other, represented by strain 20 from a blood plate and strain 22 from an acid beef-liver plate, was found as a lance-shaped diplococcus that only after several transplantations began to form any chains in broth. The colonies on blood agar were minute, surrounded by a relatively wide vivid green zone. They resembled much more nearly the characteristic alpha type found in the throats of patients with cold and influenza. It is, therefore, noteworthy that they gave the sugar reactions of Strep. salivarius. This group is supposed to be found rarely in the intestine, but is often associated with focal areas in the nasopharynx. Two strains from this source (strains 24 and 25) gave the sugar reactions of Strep. salivarius, although they continued, even after long cultivation, to grow much more delicately than the strains 20 and 22 from specimen 19. The single strain of Strep. salivarius isolated by Holman[4] from the feces was a long chain variety, in this

TABLE 1

AGGLUTINATION AND SUGAR REACTIONS OF STRAINS OF STREPTOCOCCI ISOLATED FROM INTESTINAL SPECIMENS AND NASOPHARYNX OF W.

Cultures			Agglutination Reactions with Serum of Rabbit Immunized with						Sugar Fermentations					Classification According to Holman
			Strain 1		Strain 11		Strain 9							
No. of Strain	No. of Specimen	Medium	Titer	Control Serum 1:100	Titer	Control Serum 1:100	Titer	Control Serum 1:50	Dextrose	Lactose	Mannite	Saccharose	Salicin	
1	1	Blood	1,600	0	0	0								
2	1	Blood	0	0	400	100	600	50	++	++	±	++	++	Mitis or fecalis
3	1	Cooked meat	200	0	0	0	0	0	++	++	++	++	++	Fecalis
4	1	Endo	200	0	0	0	0	0	++	++	++	++	++	Fecalis
5	1	Endo	1,600	0	0	0								
6	1	Acid beef liver	800	0	0	0	0	0	++	++	—	++	++	Mitis
7	2	Blood	0	0	0	0								
8	2	Blood	0	0	0	0	0	0	±	—	—	—	—	Ignavus ?
9	3	Blood	0	0	1,400	100	600	0	++	±	—	±	++	Equinus or mitis
10	3	Blood	0	0	0	0								
11	4	Blood	0	0	1,400	0	0	0	++	—	—	++	++	Equinus
12	4	Blood	100	0	100	0								
13	4	Blood	0	0	1,400	0	0	0	++	—	—	++	++	Equinus
14	4	Blood	0	0	200	0	100	0	++	—	—	++	++	Equinus
15	5	Cooked meat	0	0	0	0	0	0	++	++	++	+	++	Fecalis
16	10	Acid beef liver	1,600	100	0	0	0	0	++	++	—	++	++	Mitis
17	10	Acid beef liver	0	0	0	0	...	...	±	—	—	++	—	Ignavus
18	15	Blood	200	0	0	0	600	0	++	++	—	±	++	Mitis
19	15	Blood	0	0	400	0	0	0	++	++	++	++	++	Fecalis
20	19	Blood	.....	...	0	0	0	0	++	++	—	++	—	Salivarius
21	19	Whey	.....	...	.....	...	0	0	++	++	—	++	++	Mitis
22	19	Acid beef liver	.....	...	.....	...	0	0	++	++	—	++	±	Salivarius or mitis
23	25	.....	.....	...	.....	...	0	0	++	++	++	++	++	Fecalis
24	Nasopharynx	.....	.....	0	0	0	0	0	++	++	—	+	—	Salivarius
25	Nasopharynx	.....	1,600	0	0	0	0	50	++	++	++	++	++	Fecalis

The following symbols are used to indicate sugar reactions: ++, marked fermentation; +, faint fermentation; ±, doubtful fermentation; —, no fermentation.

The titer of a serum for any strain is the highest dilution showing complete or almost complete agglutination.

respect differing from ours. We tried repeatedly to isolate a similar type from subsequent specimens, but were not able to recover a strain culturally resembling the diplococcoid form of strans 20 and 22. As is well known, Poynton and Paine [5] found a diplococcus which they thought was directly associated with rheumatoid arthritic conditions.

If one now turns to the cross agglutinations which were carried out with serums from rabbits immunized against strains isolated from the first, third and fourth intestinal specimens, one finds that serum of strain 1 partially agglutinated all of the strains from the same specimen, except strain 2. It also slightly agglutinated a few strains from later specimens. Some of the strains partially agglutinated by the serum may be more closely related to the immunizing strain than appears from the result. It is probable that the agglutination in higher dilution of the immunizing strain may be due to its constant transplantation during the period of injection. Nevertheless, there are few strains that are completely agglutinated by the serums even at the dilution of 1:100. The only "fishing" completely agglutinated by serum of strain 1 was a streptococcus fecalis of nasopharyngeal origin. The two strains strongly resembled one another in morphologic and cultural characteristics. Unfortunately absorption tests could not be made because strain 1 died out during the course of the work. The relationship is suggestive, but no definite conclusions can be drawn without the confirmatory test.

Serum of strain 11 strongly agglutinated one strain from the same specimen and none from any other specimen. Serum of strain 9 was not tried on any strains from the same specimen. It strongly agglutinated one strain from specimen 15, i. e. strain 18. In order further to investigate the relationship between the two strains, an absorption test was carried out. It was found that strain 18 completely absorbed the specific agglutinins for strain 9. A rabbit was then immunized with strain 18 and reciprocal agglutination and absorption tests were made. It then appeared that strain 9 absorbed all the specific agglutinins for 18. Thus by the most delicate test at our disposal the identity of the two strains was established.

The data of the absorption tests are given in table 2. The method followed was that used in our serologic study of alpha streptococci from the throat.[2]

We conclude from this set of experiments that there must be present in the upper intestinal tract of W. at different times a considerable variety of streptococcal types. Nevertheless, the fact that we have

[5] Brit. Med. Jour., 1902, 2, p. 1414.

isolated, at intervals of 2 months, 2 strains apparently identical, may indicate either that there is a flora which is more or less permanently established in the intestinal tract or that there is a focus at some other point from which these organisms are introduced into the alimentary canal.

While it is of course quite possible that one streptococcal strain among many in the tonsils, teeth or intestinal tract may be directly responsible for the symptoms of disease, the mere presence of an unusual number and variety of nonhemolytic streptococci hardly justifies the assumption of a causative inter-relationship of any one or all of them to the pathologic condition, especially in view of the fact that these bacteria are common secondary invading organisms. N. and J. Mutch [3] suggest that "a negative phase in the immunity reactions of the body, determined by increased bacterial activity at one focus, affords bacteria of allied race, opportunity of gaining simultaneous advantage over the cells of distant tissues among which they live."

TABLE 2

AGGLUTINATION BY SERUMS 9 AND 18 BEFORE AND AFTER CROSS ABSORPTION

	Strain 9	Strain 18
Serum 9	1,000	1,000
Serum 9 treated with strain 9	0	0
Serum 9 treated with strain 18	0	0
Serum 18	1,000	1,000
Serum 18 treated with strain 9	0	0
Serum 18 treated with strain 18	0	0

In view, however, of the recognized adaptability to mucosal surfaces of the nonhemolytic streptococci, the establishment of members of this group in the small intestine does not necessarily seem to imply the existence of a pathologic flora, any more than does the extreme predominance of the colon group at lower levels of the intestine. It seems plausible to regard the streptococci, when present in large numbers, as indications of deranged intestinal functioning or of excessive amounts of carbohydrate in the diet rather than as evidence of the existence of a primary focus of infection with production of toxic substances or with tissue invasion. Lactose feeding, we found, stimulated this group, and it has been affirmed that the presence of carbohydrate associated with loci of stagnation is the most potent factor in establishing its predominance [3] just as undigested proteins are supposed to favor the proteolytic organisms. It seems probable that an adaptation of particular bacterial

types to the metabolic conditions within the tract of each person may gradually develop, until certain individual strains become indigenous.

Several strains of intestinal streptococci were tested for direct agglutination by the serum of the patient W. using normal serum as control. As the patient had some months earlier received an autogenous vaccine of intestinal streptococci, positive reactions were expected (table 3).

In the first test, we used 1 : 100 as the highest dilution, but when it was found that in all lower dilutions a prezone developed, we repeated the test with higher dilutions, using in the case of the patient some serum that had been held over for 3 days.

TABLE 3

AGGLUTINATION TESTS BY WALSH AND GOLDMAN SERUMS OF STRAINS OF INTESTINAL STREPTOCOCCI

Number of Strain	Serum	Titer
20	W	3,200
	G	1,600
11	W	100
	G	100
18	W	3,200
	G	400
9	W	3,200
	G	1,600
4	W	100
	G	100
25	W	0
	G	0
Burke	W	1,600
	G	800

Agglutinations were carried out on 2 separate days; the first set was carried through a 1:100 serum dilution. The second set was carried out with 1:100 as the lowest serum dilution.

It was found that both the patient's and the control serum agglutinated most of the strains in high dilution. The patient's serum was the more potent, but the difference seems of little significance. This was the case not only when the serum was tested with autogenous strains, but also when tested with a strain isolated from the specimen of another subject (B.). The test with the streptococcal strain of nasopharyngeal origin was negative. Unfortunately it was not possible to test it with as high a dilution of serum as was used for the other strains.

These results prove how misleading the mere fact of a high titer agglutination may be. They also indicate that since human serum appears to have developed a high antibody content against intestinal

streptococci, these organisms must be regarded, as indeed the flora of the feces would lead us to suppose, as normal inhabitants of the intestinal tract.

SUMMARY

The same strain of nonhemolytic streptococcus, identified by agglutinin absorption methods, was twice recovered from the intestinal contents of one patient, at an interval of two months.

The persistence of this strain may indicate a well adapted flora, native to the intestinal tract of the person from whom the strain was isolated or a focus at a higher level from which the strain was at intervals disseminated.

Normal human serum appears to contain a high antibody content of group agglutinins for some nonautogenous streptococci from the small intestine.

HEAT RESISTANCE STUDIES

I. A NEW METHOD FOR THE DETERMINATION OF HEAT RESISTANCE OF BACTERIAL SPORES

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A method for determining the thermal death points of heat resistant spores at temperatures above 100 C. was described by Bigelow and Esty.[1] In this article results are published to show the relationship of time to temperature from 100 to 140 C. of spores of 15 typical thermophilic organisms, the influence of numbers and the effect of varying hydrogen-ion concentration in the same and different solutions on the time necessary to sterilize them. Since then these studies have been greatly extended and the effect of other variable factors investigated. Some of these factors are briefly discussed in the summary report on the heat resistance of the spores of B botulinus and allied anaerobes,[2] such as the variation of different strains and of individual spores of the same strain, the influence of the chemical composition and the reaction of the mediums used for the production of spores, the effect of drying, age, different concentrations of sodium chloride and the variation in the heat resistance of spores in food juices, peptone solutions, etc.

In the course of this investigation on the spore resistance of B. botulinus and certain heat resistant organisms from spoiled canned foods, our former views were changed in certain respects, and the original method had to be modified in the light of the more recent developments.

This new method furnishes an explanation for certain irregularities observed in our results heretofore and gives more accurate information as to the actual resistance of organisms to moist heat. Results by this method can be directly applied to the sterilization of canned foods and are of utmost importance in determining the maximum resistance of B. botulinus spores in order that safe sterilizing procedures can be established for the preservation of foods by heat.

SINGLE TUBE METHOD

A brief statement of the procedure followed in the former tests will recall the underlying principles involved and serve as a basis for

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[1] Jour. Infect. Dis., 1920, 27, p. 602.
[2] Ibid., 1922, 31, p. 650.

comparison of the one to be described here. A definite amount of the spore suspension is inoculated into a series of sterile tubes of standard size, approximately 7 mm. inside diameter with 1 mm. thickness of wall. These inoculated tubes are sealed in an oxygen flame, preheated in boiling water and then completely immersed in an electrically heated oil bath, so adjusted and controlled as to maintain a constant and uniform temperature throughout. At definite periods of time a single tube is removed, cooled immediately in a bath of ice water and subcultivated in a favorable medium, if not already in suitable material, and incubated to determine growth. The time intervals of heating are so spaced as to define the resistance within close limits. This method is termed the single tube series and will be referred to as such. It has been used quite generally by workers during the last 3 years, especially by those studying the resistance to heat of the toxin and spores of B. botulinus. At least 200,000 spore suspensions are known to have been heated according to the principles of this method.

An analysis of our data on the heat resistance of spores over a period of 5 years reveals a consistent and common occurrence of "skips" in a series of single tubes heated for different times. Cultures heated for periods up to and including a certain time show growth uniformly when incubated in a favorable environment, but for longer periods the results may be irregular and inconsistent. Spore suspensions in a single tube series may occasionally contain viable organisms that have been heated for a considerable time in excess of those in which uniform growth has been observed, even though several tubes may have been found to be sterile when heated for a much shorter time. This variation in the results has occurred in all of our work on spore resistance including strains of the common aerobic soil and water organisms, facultative and obligate anaerobic thermophiles that have been isolated from underprocessed canned foods and B. botulinus and allied anaerobes.

At first the presence of "skips" was considered to be due probably to faulty technic or to the failure to inoculate the recovery medium with the surviving spores that withstood the heating. However, cultures heated and incubated in the same sealed tubes in which they were heated without subcultivation gave similar results. In such cases the entire heated material was incubated, and the survival of any spores should be indicated by growth if the medium is sufficiently sensitive for the germination of a single organism. Tables 1 and 2 present typical examples of this variation in the heating of a definite number of spores in different solutions at 110 and 115 C.

TABLE 1

COMPARATIVE HEAT RESISTANCE OF SPORES OF ORGANISM 1518 IN DIFFERENT SOLUTIONS AT 110 C. SINGLE TUBE VS. PERCENTAGE SURVIVAL

Spores Suspended	Time in Minutes at 110 C.																										
	5	8	10	12	13	15	17	18	20	22	25	27	30	32	35	37	40	42	45	47	50	52	55	57	60	62	65
Phosphate mixture			100	96.4		64.3		42.9	39.4	18.9																	
P_H 5.10	+	+	+	+	+	+		+	—																		
100,000	+	+	+	+	+	+		+	—																		
Phosphate mixture						+	96.7		44.4	40	29.6	8.1	3.7		7.7												
P_H 5.55						+	—		—	+	—	+	—		—		—										
100,000						—	+		+	—	—	—	—		—		—										
						—																					
Phosphate mixture															80		37.2		25		17.5						
P_H 6.20											+		+	—	+	—	—	+	—		+						
100,000											+		—	+	—	+	+	+	—		—						
Phosphate mixture																					88.5				37		
P_H 7.09															+		+	+	+	+	—	—	—		—		
100,000															+		+	+	+	+	+	—	—		—		
String bean juice														93.3	93.3		22.2										
P_H 5.21													+		+	—	—	—	—	+	—		—		—		
100,000													+		+	+	—	—	—	—	—		—		—		
Pea juice																							74.1				
P_H 5.79																	+		+	+	+	+	+	+	—		
10,000																	+		+	+	+	+	+	—	+		
Corn juice																							96				26.9
P_H 6.10																	+		+	+	+	+	+	+	+	+	+
10,000																	+		+	+	+	+	+	+	+	+	—

It will be seen in the single tube series in table 1 that growth as indicated by a plus sign is uniform for a certain time, for example, when 100,000 spores of organism 1518 are suspended in a phosphate solution P_H 5.10, uniform growth results for 18 minutes at 110 C., in P_H 6.20 for 25 minutes and in P_H 7.09 for 47 minutes, while 10,000 spores of the same organism suspended in corn juice P_H 6.10 showed uniform growth for 62 minutes. After these times "skips" are noted, and in some cases cultures heated for relatively long periods show growth. Note the results obtained in phosphate P_H 5.55 in which skips occur up to 30 minutes; in phosphate P_H 6.20 one culture survived for 50 minutes, and in string bean juice P_H 5.21 although

TABLE 2

COMPARATIVE HEAT RESISTANCE OF SPORES OF ORGANISM 1518 IN DIFFERENT SOLUTIONS AT 115 C. SINGLE TUBE SERIES VS. PERCENTAGE SURVIVAL *

Spores Suspended	Time in Minutes at 115 C.															
	4	6	7	8	9	10	12	14	15	16	17	18	20	22	24	27
Corn juice									96.0		61.5		26.9	19.2		
P_H 6.1						+	+		+		—		—	—	—	—
10,000						+	+		—		—		—	+	—	—
Pea juice								75.0		62.5		4.2	4.3			
P_H 5.79							+	—	—	—		—	—	—	—	
10,000							+	—	—	—		—	—	—	—	
Phosphate								60.0		28.0		4.0	0.0			
mixture			+			+	—	—	—	+		—	—			
P_H 7.09			+			+	+	—	+	—		—	—			
100,000																
Phosphate					12.0	4.2	0.0		0.0							
mixture					—	—	—		—							
P_H 6.20	+	—	—	—												
100,000	+	+	—	—	—	—	—		—							

* The figures indicate survival based on 25-26 tubes heated alike.

uniform growth ceased after 35 minutes, yet one tube showed growth after being heated for 47 minutes. Table 2 shows the same phenomenon, although it will be noted that fewer "skips" occur. This is due to the fact that the range in resistance is less, since a minute at 115 C. has greater sterilizing value than a minute at 110 C.

Attention is also directed to the variation of the same suspension when duplicate tubes are heated, as shown in the same tables. It will be observed that up to a certain point both cultures are viable, after which skips are quite frequent. This same variation was observed in different solutions and at all temperatures. It becomes still more confusing if three or more similar suspensions were heated for the same time. Skips are more frequent if the time intervals are short

than if the times are widely separated, owing to the fact that beyond a certain point only an occasional tube will contain viable organisms. This is also true of lower temperatures in cases in which it requires several hours as compared to a few minutes at a much higher temperature.

These "skips" are now accounted for on the ground of variation in the resistance of individual pores in any suspension, since they occurred in cultures containing different numbers of spores and to some extent in all mediums.

MULTIPLE TUBE METHOD

To determine the extent of this variation and its effect on the ultimate spore destruction, a series of experiments were made in which different numbers of tubes all containing the same suspension were heated for different times within the range of the skips. In this work spores of our organism 1518 were selected because of the high degree of heat resistance and the rapid germination of viable spores of this organism after heating. This organism produces much acid and no gas in carbohydrate mediums and especially in canned corn and peas. It was isolated originally from a can of sour corn. Similar results to those obtained on this organism have been found with spores of B. botulinus and those of thermophilic bacteria. In these tests special care was exercised to obtain as uniform a spore suspension as possible, free from clumps or foreign material.

Harrison and Hood [3] found that the thermal death point depended on the presence or absence of clumps of spores. A comparison of the results from the filtered material with those obtained without filtering the spore suspension shows the serious effect of clumping in raising the thermal death points of spores. They believe that the central spores in the clumps are greatly protected. Organisms from filtered material having a thermal death point ranging from 1 to 10 minutes in boiling water were found to survive from 30 minutes to 3 hours if unfiltered. An increase of several minutes in the thermal death points of individual spores greatly prolongs the time required for the destruction in clumps. It is also stated that although thermal death points were determined several times absolutely consistent results were not obtained. Occasionally variations of one or two minutes occurred which were accounted for either by a difference in the concentration of spores or by the individual variation of the spores of one organism in their power to resist heat.

[3] Transactions of Roy. Soc. of Canada, 1923, 17.

PREPARATION OF SPORE SUSPENSION

Several Kolle flasks containing nutrient agar were each inoculated with 2 c c. of a stock spore suspension of a pure culture of the organism to be tested (in this case organism 1518). If no stock spore suspension was available, growth from 2 or more agar slants was suspended in a small amount of sterile distilled water and the resulting suspension used as the inoculum. This suspension was distributed uniformly over the surface of the agar in the Kolle flasks and incubated under optimum conditions (55 C.) for 48 hours. At the end of this incubation period, the bacterial growth in each flask was washed off by the addition of a little sterile distilled water, and the suspension was removed to a sterile flask. Care was taken not to cut the agar and mix any fragments with the bacterial suspension. This resulting suspension was thoroughly shaken and placed again at 55 C. for another 24 hours. Smears from the 48-hour cultures showed numerous vegetative organisms and some spores, while stained preparations after the second incubation showed an abundance of spores and fewer vegetative cells. This final suspension was again thoroughly shaken, filtered through a heavy layer of sterile cotton, heated to 90 C. for 10 minutes to kill the vegetative forms and again shaken. The suspension was then counted and placed in sterile ampules, which were sealed, and held at icebox temperature as the stock on which the heat resistance tests were made. If the suspension was not sufficiently concentrated, it was centrifuged, the supernatant liquid decanted and the spore sediment resuspended and made up to the desired concentration. In some cases, a sand filter was also used and the suspension shaken with sterile sand or glass beads. Plate counts made in triplicate according to standard methods on different suspensions that had received both the above treatment and that filtered through cotton alone showed uniform results without any evidence of clumps. Furthermore, heat resistance tests on these suspensions prepared by the different methods of filtering showed no indication of variation in this respect.

After vigorous shaking of the spore suspension in the sealed ampule, the tube was opened aseptically, and definite amounts were inoculated into solutions of different chemical composition such as M/15 phosphate solution of different P_H values (Sorenson mixture of Na_2HPO_4 and KH_2PO_4), nutrient broth, P_H about 7, and certain food juices, notably canned corn and peas. The concentration of the spores in these solutions was so adjusted that only 1 or 2 c c. of the

stock spore suspension was added to each 100 c c. of the solution to be heated. In this way the inoculum did not affect the composition of these solutions appreciably nor was the P_H value altered. The results thus obtained apply to the conditions under which the spores were heated. After thorough shaking, 1 c c. of these suspensions was inoculated into sterile tubes similar to those used formerly, immediately sealed and heated as usual. The inoculation of these tubes was made from a sterile 10 c c. pipet graduated to 0.1 c c., and the sealed tubes were held in ice water until heated. The heating of the cultures was carried out as described in the other method. The temperature readings were taken from a Beckmann thermometer and the heat controlled by means of a Sligh thermo-regulator. The temperature was kept uniform by means of a turbine stirrer and constant movement of the tubes by hand. Cultures in phosphate solutions were always subcultivated. In the other cases the heated solutions served as culture medium for growth; accordingly, whenever possible the sealed tubes were incubated and growth observed by turbidity and confirmed by production of acid.

The germination of heated spores of this organism (No. 1518) was not retarded, for surviving cultures usually showed growth in from 24 to 48 hours when incubated under optimum conditions. Occasionally growth has been observed on the 3rd to 5th day. Cultures showing no growth during this period have remained sterile indefinitely. All cultures were incubated for at least from 7 to 10 days, while a large number have been held over a month.

Growth is confirmed by acid production which is determined either by adding 2 c c. of the stock brom-cresol purple indicator (0.4%) to each 100 c c. of the solution containing the spores in which they can grow at the time the solution is inoculated or by emptying the heated contents of the tube after sufficient incubation into 0.4% of the indicator solution. Subcultures from the phosphate solutions were made in dextrose broth P_H 7, to which had been added 0.4% of brom-cresol purple indicator.

The addition of this indicator to the solution to be heated does not appear to inhibit the development of viable organisms (1518) but it does affect slightly the hydrogen-ion concentration of the solution and the heat resistance. This phase will be discussed further in a subsequent paper. In view of the fact that material in which spores of this organism have grown and produced acid may show no viable organisms after the 3rd day of incubation, and after the 4th day consistently no growth, making recovery of this organism impossible,

it is essential that some criterion be found to determine whether or not growth did occur. On this basis heated cultures of acid-producing organisms should be tested for acid production by using brom-cresol purple indicator either in the medium used for subculturing or by emptying the contents into this indicator solution after sufficient incubation. These methods give clear-cut results and afford an easy means for determining the growth of this organism.

EXPERIMENTAL

Typical results of heating tests based on 25 to 30 tubes each containing the same suspension all heated alike for the times given are shown in tables 1 and 2. It will be seen that when a large number of tubes are heated a single tube series has limited value. For example, table 1 shows that spores in a phosphate solution P_H 5.1 in single tubes were viable for 18 minutes at 110 C., whereas even after 12 minutes one of 26 tubes failed to show growth, or 96.4% survived; after 15 minutes 64.3% survived, and in 20 minutes single tubes indicated complete destruction, but 39.4% were still viable by the multiple tube method.

Cultures in pea juice P_H 5.71 according to the single tube series, even in duplicate, indicated complete survival after 55 minutes' heating at 110 C., while of 26 tubes all containing the same suspension and heated for the same time, only 74.1% proved to be viable, and in corn juice P_H 6.10 duplicate tubes showed uniform growth up to and including 62 minutes at 110 C., while 1 of 25 tubes was sterile after 55 minutes and only 26.9% survived 65 minutes. However, the duplicate single tube series indicated 50% survival for this period.

It will also be seen that in general the percentage survival greatly decreases as the heating progresses. Occasionally, however, it is found that a somewhat higher percentage may be obtained after a longer heating. This has been observed in particular when the percentage is very low, about 5 or less, when only an occasional spore remains, and then only in one of a large number of tubes. This same condition is often present when only a few tubes (say 5 or 10) are subjected to the same treatment and the time intervals are very short. A difference of one positive in such cases would show considerable irregularity.

The percentage survival of different numbers of spores in different mediums has been determined on varying numbers of tubes all heated alike as follows: 2, 6, 10, 25, 30, 60, 100 and 300 tubes. The results show uniformity in proportion to the number of tubes heated, the larger the number the closer the range obtained in duplicate sets. The time intervals must be carefully selected in order that significant differences in the percentage may be obtained. Tables 1 and 2 also show a difference in the heat resistance in different mediums. Solutions of the same P_H value have different heat resistances, for example, table 1 shows that phosphate solutions of P_H values 5.1 and 5.55 as compared to string bean juice P_H 5.21 and pea and corn juices with P_H values 5.79 and 6.09, respectively, to phosphate P_H 6.2 require different times to sterilize under similar conditions. In the latter case different numbers of spores were also heated which cause an added effect.

If these percentage survivals recorded in tables 1 and 2 are plotted on semi-logarithmic paper against time, the general direction of the curve is determined. Charts 1, 2 and 3 are graphic illustrations of this. A straight line is drawn as nearly as possible through the experimentally determined points. The per-

centages are based on results obtained on heating 25 to 30 tubes each, which may be subject to some experimental error. It is felt, however, that such curves are justifiable and give the general direction within close limits. By means of such curves it is possible to state the approximate percentage survival on

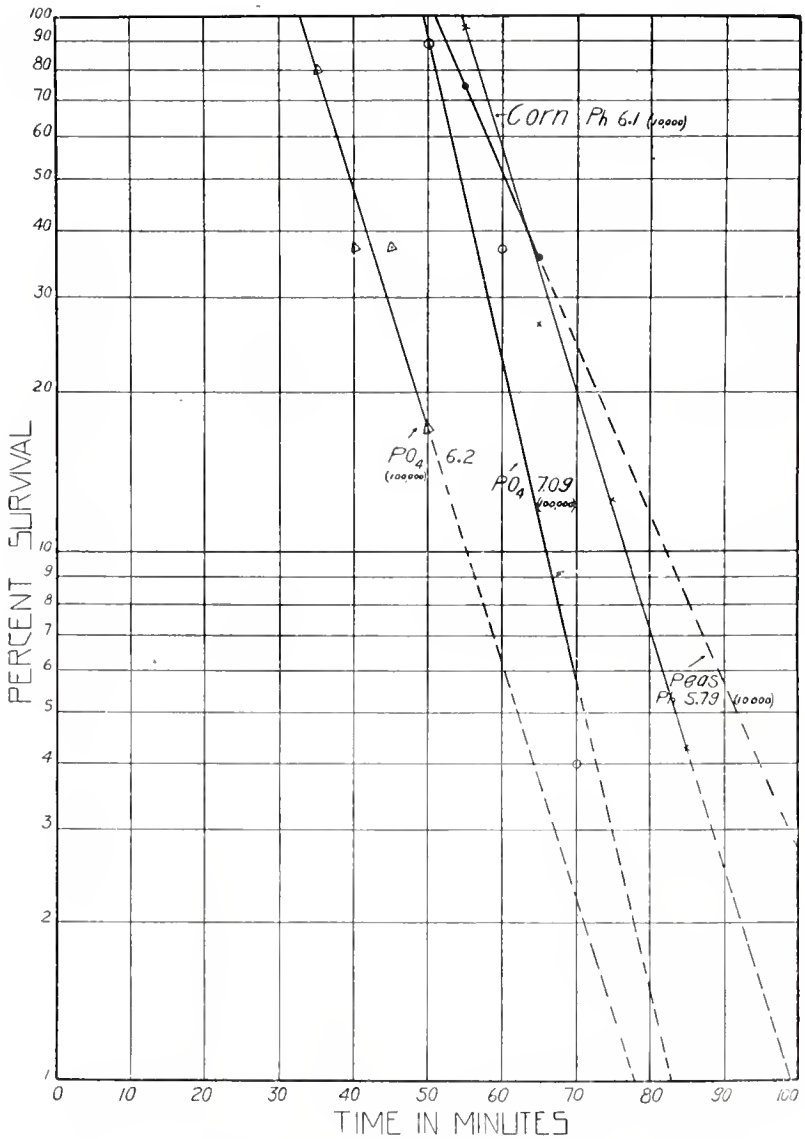

Chart 1.—Heat resistance of 1518 at 110 C. in different solutions.

that particular suspension in the heated solution for any heating period, and determine certain thermal death time relations. These curves are suggestive of a logarithmic nature similar to that already found in the death rate of spores and in thermal death time relations as pointed out by Bigelow[4]. The

[4] Jour. Infect. Dis., 1921, 29, p. 528.

experimental error as seen by an inspection of the charts is very slight in most cases, the line coming within one or two minutes of the experimentally determined times for a certain percentage survival.

Chart 1 shows graphically the heat resistance of organism 1518 at 110 C. in phosphate solutions P_H 7.09 and 6.2, corn juice P_H 6.1 and pea juice P_H 5.74

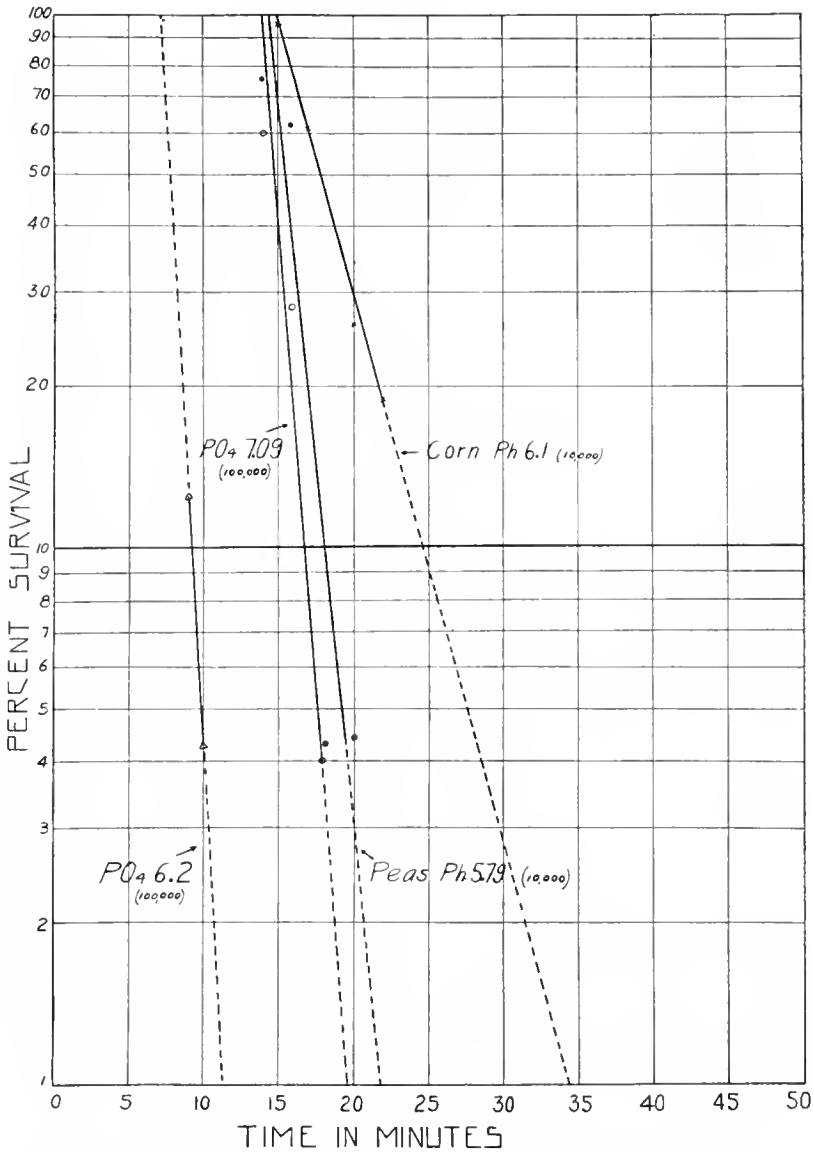

Chart 2.—Heat resistance of 1518 at 115 C. in different solutions.

according to the precentage survival. It will be seen that the curves are not parallel to each other, indicating that there is no definite relationship in the resistance to different solutions. However, the two phosphate solutions appear to be related. This is more clearly seen in chart 2 and especially in chart 3 in which curves of 4 phosphate solutions are plotted. The phosphate P_H 7.09 curve

in chart 1 has been drawn to include the 4% survival points while in chart 3 this curve has been drawn through the two upper points only. A similar relationship is shown in the effect of numbers of the same solution and will be discussed in a later paper. In the light of these findings it is suggested that a standard type curve be established for a given number of spores in a given solution by

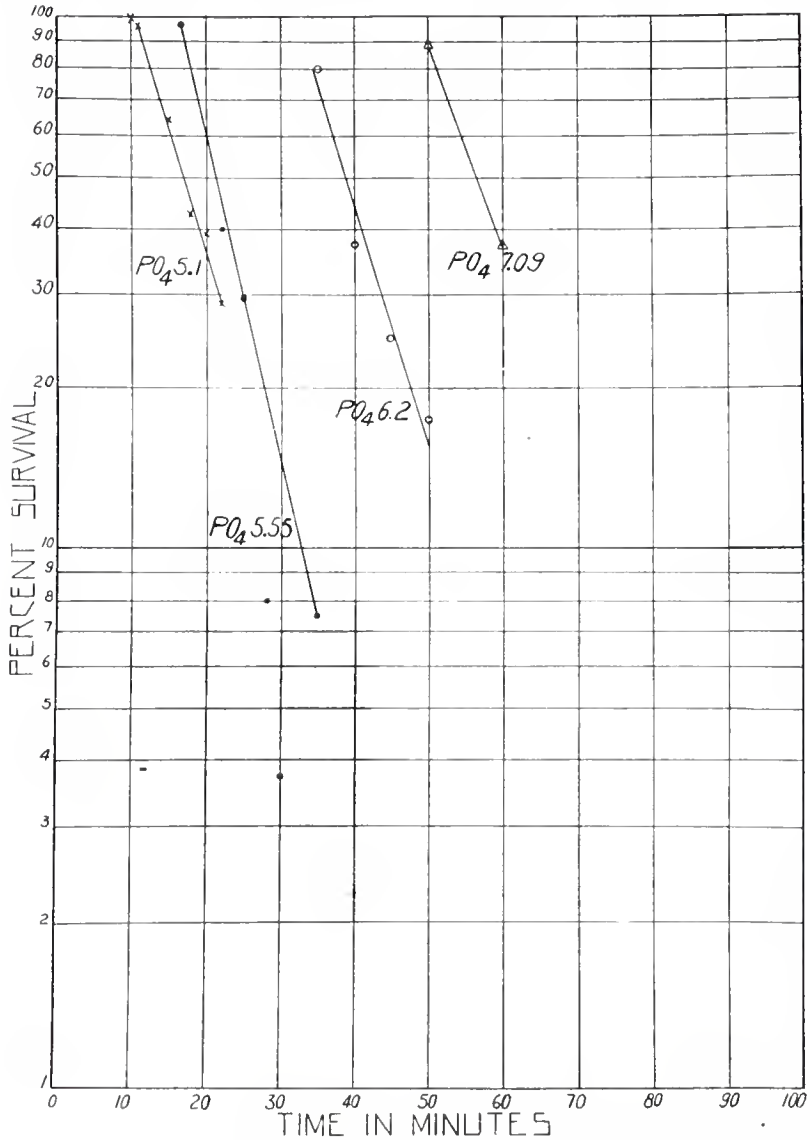

Chart 3.—Heat resistance of 100,000 spores 1518 at 110 C. in phosphate solutions.

determining percentage survivals for at least 4 well selected times on a large number of tubes, 100 to 300 each time. Such a curve could then be used as a type for determining the effect of different numbers of spores in the same solution or the influence of varying hydrogen-ion concentration in phosphate solution by constructing a parallel curve through the percentage survival point that has been definitely fixed on, say 300 tubes.

According to the logarithmic charts, the lower limit of the precentage survival scale has been arbitrarily assigned the value of 1. Consequently the upper limit is 100. It is realized that in a strict mathematical sense these values are not correctly represented, but for our purpose to show the range in the resistance as indicated by the charts such a scale seems to be justified.

Any point becomes more nearly correct when the percentage survival is based on larger numbers of tubes, as previously stated. It is believed, however, that for all practical purposes 30 tubes of the same suspension heated for at least 4 different times that are well selected give reliable and fairly consistent results. Such percentage survivals are far more significant and valuable than those obtained on single tube tests, or when from 2 to 5 tubes are used. Single tube results are valuable in preliminary tests to locate the range within which all but the occasional spore is destroyed. A few tubes all heated alike for well selected times determines the approximate percentage survival and serves to establish the range of maximum resistance. Such a procedure is highly recommended for such purposes. The actual death point or the maximum resistance of the more resistant spore is determined only by heating a large number of tubes for different times.

Based on the percentage survival or multiple tube method as differentiated from the single tube series the following relations have been studied extensively: The time-temperature, the effect of hydrogen-ion concentration and numbers, the effect of heating different numbers of tubes to establish a proper percentage survival, the effect of heating spore suspensions in different mediums with especial attention to food juices and the determination of processes for different canned foods as determined in tubes heated according to different heating curves and in cans experimentally inoculated with similar spore suspensions processed for different times at different temperatures according to previously determined results in tubes.

The tube results were obtained by heating a large number of tubes according to definite heating curves characteristic of the rate that the heat penetrates the center of certain canned foods. In this way the spore destruction was accomplished by a progressively rising temperature and not at a constant temperature. This data is being analyzed and prepared for publication.

All of the foregoing factors are essential to a proper understanding of the spore resistance to varying amounts of heat in different solutions and have a direct bearing on the sterilization of canned foods. Without a knowledge of the underlying principles operative in the destruction of bacterial spores it is impossible to state definitely the processes necessary to sterilize any product, food material or other material, even though all the other factors which affect them are well established.

It has been definitely shown that the individual spores in a given suspension vary in their heat resistance. Heating a suspension for a certain time destroys all but a few resistant individuals which may require a somewhat longer exposure to kill. On account of such variability, "skips" are bound to occur and irregular results will be obtained if single tube series are employed. Recognition of this fact explains why the occasional can or jar of preserved food has been responsible for botulism outbreaks, why occasionally a batch of culture mediums, glassware, etc., is not sterile and why at certain times infections in hospitals are encountered. The necessity of determining the thermal death points according to the percentage survival based on a large number of tubes cannot be over-emphasized if safe sterilization measures are to be employed. Such a procedure affords a more accurate and reliable means than the single tube method.

CONCLUSIONS

A new method for determining the thermal death points of heat resistant spores is described. The principle involved is the heating of a large number of tubes, at least from 25 to 30, each containing the same suspension and all heated alike for at least 4 different times that are well selected in order to cover the entire range of heat resistance based on percentage survival.

Experimentally determined points according to this method when plotted on semilog paper suggest logarithmic curves.

The larger the number of tubes heated for any time, the more accurate the percentage survival for that specific heating period.

Individual spores vary in their heat resistance.

The heat resistance of bacterial spores is not a constant but depends on a large number of variables. Any statement regarding it should include all the conditions under which the spores are produced, heated and subcultivated.

This new method has a direct bearing on all sterilization processes, since the actual (resistance) death point can be more accurately determined.

STUDIES ON THE IDENTIFICATION OF MONILIA PSILOSIS

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The difficulty of differentiating Monilia psilosis from certain other fungi which are present in normal stools, by cultural methods, was recently brought to my attention when I was unable to identify a yeast-like organism found in the stools of a patient who had been sent from China to America to recover from tropical sprue. The further study was aided by Dr. Bailey J. Ashford, Porto Rico, and Dr. J. M. Rogers and Dr. L. C. Todd, of Charlotte, N. C., who sent cultures of Monilia psilosis isolated from sprue patients. The comparison of these organisms with strains of fungi from the feces of patients who did not have sprue and with two strains of so-called blastomycetes from blastomycosis, is the basis of this study. The colonies of many of the wild yeasts and molds on Sabouraud's medium can be distinguished readily, and therefore only the colonies which seemed to resemble Monilia psilosis are included in the study. The methods used are those emphasized by Ashford and included a study of the morphology; growth in gelatin, liquid and solid mediums; reaction in litmus milk and various sugar mediums; pathogenicity for laboratory animals and agglutinin production in rabbits.

Growth on Sabouraud's Agar (4% maltose, plus 2 acidity).—Ashford [1] describes the typical colony of Monilia psilosis as a faint greenish, creamy, soft, elevated growth with clearly defined borders. He believes that the faint greenish tinge is not present in yeasts other than Monilia psilosis, although in some strains it is absent. I was unable to detect this peculiarity of color, probably due to the age of the culture when received. All of the "wild" yeasts produced creamy or white, elevated sharply defined colonies.

Morphology.—Typical Monilia psilosis is described as a large, round, bright, clear-cut slightly granular, nucleated yeast, from 4 to 7 microns in diameter, which frequently shows marked variability in size and shape. The "wild" yeasts in my series were generally smaller, from 2

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[1] Am. Jour. Med. Sc., 1917, 154, p. 157.

to 4 microns in diameter, while Monilia psilosis varied from 3 to 6 microns. However, one strain from the stools of a case of tuberculous enteritis was as large as Monilia psilosis. Mycelial elements and side buds were observed in all strains.

Growth in Liquid Medium.—There was marked variation in the appearance of all strains in dextrose broth at different intervals after inoculation. The sediment was abundant in all. A collar was present in five wild strains and all of the strains of Monilia psilosis. A pellicle was present in one of the wild strains and absent in all the others.

Growth in Gelatin.—Ashford ascribes considerable importance as a diagnostic aid to the inverted pine tree appearance, produced by the fine, long, hairlike, mycelial extension into gelatin which takes place along the line of the stab. The wild yeasts in this series produced a short, brushlike extension that gave an entirely different appearance from that produced by Monilia psilosis. However, the strains of blastomycetes produced a fine long mycelial extension, similar to but more exaggerated than that produced by Monilia psilosis.

Reaction in Litmus Milk and Sugar Mediums.—One per cent. sugar broths, with the reaction adjusted to plus 1 acidity and containing Andrade's indicator, were incubated at 38 C. for 8 days. The results given in table 1 reveal a variation in the reaction of different strains of Monilia psilosis. Ashford has found that Monilia psilosis when first isolated ferments glucose, levulose, maltose and sometimes saccharose and galactose. He believes that a strain of Monilia psilosis which fails to ferment maltose is degraded, and this may account for the failure of Y-1 in this respect, because Robers [2] in his description of this organism, records it as a maltose fermenter. Ashford concludes that fermentation tests alone are insufficient to decide the species.

Animal Inoculation.—Small guinea-pigs were given 2 c c. of a 24-hour dextrose broth culture intraperitoneally at intervals of 2 days, 4 times. The animals receiving the strains of Monilia psilosis died 14, 11, and 6 days, respectively, after the initial injection. In the peritoneum liver and spleen of these animals, there were mycotic abscesses from which pure cultures of yeasts were obtained; 2 c c. of a 24-hour culture of these organisms recovered from the guinea-pig were injected into 3 more animals, and death occurred in all 3 within 48 hours.

Seven wild strains, injected in a similar manner, did not cause death except in one animal injected with Y-15 (from a case of amebic dysen-

[2] Jour. Am. Med. Assn., 1922, 79, p. 1677.

TABLE 1

REACTIONS IN SUGAR MEDIUMS

	Yeast 1 Sprue, Rogers and Todd	Yeast 17 Sprue, Feces, Ashford	Yeast 18 Sprue, Tongue, Ashford	Yeast 2 Stool of Man Fed on Lactose	Yeast 3 Man on Normal Diet	Yeast 5 Stool of Enteritis	Yeast 6 Normal Stool	Yeast 7 Tuberculous Enteritis	Yeast 13 Tuberculous Enteritis	Yeast 14 Former Sprue Patient	Yeast 15 Amebic Dysentery	Yeast 16 Blastomycosis	Yeast 19 Blastomycosis
Litmus milk	0	Alkaline	Alkaline	Alkaline	0	0	0	Slightly alkaline	Alkaline	Slightly alkaline	Alkaline	Alkaline	Alkaline
Dextrose	0	Acid, gas	Acid, gas	Acid gas	0	Acid, gas	0	0	Acid, gas	0	Acid, gas	Acid, gas	Acid, gas
Saccharose	0	Acid, gas	Acid, gas	0	0	0	0	Acid, gas	Acid, gas	Acid	0	Acid, gas	Acid, gas
Maltose	0	Acid, gas	Acid, gas	Acid, gas	0	0	0	0	Acid, gas	0	0	0	Acid, gas
Lactose, Salicin, Inulin, Mannite, Dulcite	0	0	0	0	0	0	0	0	0	0	0	0	0

tery), that died 21 days after the first injection. There was a generalized peritonitis with no abscess formation in the liver or spleen. Cultures gave staphylococci and yeasts, but pure cultures of the latter did not kill another animal when injected in a similar manner. Neither culture of the blastomycetes had any pathogenic effect, although this is not unusual for old cultures of this organism. It is evident from these experiments that even old yeasts which have been isolated from patients with tropical sprue have pathogenic properties for guinea-pigs that are not possessed by young cultures of wild yeasts or even old cultures of blastomycetes. Dold,[3] however, has injected monkeys, dogs, and mice with wild yeasts, and obtained mycotic lesions similar to those produced by the organisms from sprue.

Agglutinin Production.—An attempt was made to produce agglutinins in rabbits by injecting, intravenously, the 48-hour growth of Monilia psilosis agglutinated every strain except one wild yeast strain solution, after heating at 60 C. for 2 hours. Injections were given at intervals of a week, and the serum was tested 5 days after the 5th injection. Agglutinins were not produced by this method but were produced readily when the sediment from old dextrose broth cultures, washed twice with normal salt solution and suspended in about 20 times its volume of salt solution, was used. Four intravenous injections into rabbits of 2 c c. of a strain of Monilia psilosis (yeast 17) and 2 c c. of a strain of blastomycetes (yeast 16) at 4-day intervals produced antiserums with ample agglutinins for the homologous strain. Each antiserum was tested against 2 strains of Monilia psilosis, 2 strains of blastomycetes and 4 strains of "wild" yeasts. To eliminate the spontaneous agglutination which usually occurred with these suspensions, a thick suspension was centrifuged at high speed for one minute and the upper portion withdrawn with a pipet. Nearly always, this made a homogeneous suspension that did not agglutinate spontaneously in the test. Equal parts of serum, diluted from 1:10 to 1:640, and suspensions of organisms in salt solution were mixed, incubated for 2 hours at 38 C., and allowed to stand at room temperature for 18 hours before the final reading was made. Normal rabbit serum, also diluted from 1:10 to 1:640, was tested against each strain as a control. One of the Monilia psilosis strains agglutinated spontaneously in every instance, despite repeated centrifugations. In table 2 the results show that the antiserum for blastomycetes agglutinated every strain tested. The antiserum for

[3] China Med. Jour., 1917, 31, p. 387.

Monilia psilosis agglutinated every strain except one wild yeast strain (yeast 2). Absorption tests were then made with each antiserum. Complete absorption of antibodies for the homologous strain was made by adding to the antiserum, diluted 1:10, an equal amount of a very heavy suspension of the homologous strain. The absorbed serum was then tested against the same strains previously used, and the absorption of the agglutinins by a strain of Monilia psilosis removed agglutinins for the wild yeast strains but not for the strains of blastomycetes, and the absorption by a strain of blastomycetes also removed agglutinins for the wild yeast strains but not for Monilia psilosis. The results of these agglutination experiments seem to indicate an immunologic relation between yeasts from sprue, blastomycosis and other sources. The failure

TABLE 2

AGGLUTINATION

Yeasts	Antiserum for Yeast 17	Antiserum for Yeast 16	Antiserum for Yeast 17 Treated with		Antiserum for Yeast 16 Treated with	
			Yeast 17	Yeast 16	Yeast 16	Yeast 17
17	320	160	0	160	80	0
1	160	40	20	160	20	20
16	160	640	80	0	0	160
19	160	160	160	0	0	80
2	0	80	0	0	0	0
5	320	160	0	0	0	0
13	160	40	0	0	0	0
15	40	80	0	0	0	0

of sprue yeasts and blastomycetes to absorb completely agglutinins for the opposite strain might indicate a degree of specificity.

An attempt to obtain a satisfactory extract of the yeast organisms for precipitin production was unsuccessful.

DISCUSSION

Monilia psilosis is a name which implies the existence of a fungus of the genus Monilia which is peculiar to the disease psilosis or tropical sprue. It is generally conceded that there is a great deal of confusion in the classification of certain classes of fungi and particularly of the genus monilia. Thus, there is a difference of opinion whether the fungus present in the lesions of thrush is a monilium or a oidium. This is probably due to the pleomorphism which all fungi present under varying conditions. Another striking example of this pleomorphism is found in the fungi which cause blastomycosis. There is a tendency among botanists to designate as blastomycetes, the budding forms of

any fungus, and as the presence of budding forms has a wide distribution among nonpathogenic fungi, they regard the term blastomycetes as incomprehensive. Castellani and Chambers [4] say that the fungus which causes blastomycosis may belong to the genera saccharomyces, cryptococcus, coccidioides or monilia. Such genera are usually so designated because of some morphologic peculiarity. For example, monilia is supposed to refer to the "chain of beads" formed by the presence of large nuclei in the hypha. Ashford points out that this peculiarity is often seen, but he does not state that it is present in all strains. It seems that any morphologic feature sufficiently important to decide the genus, should be present constantly. The morphologic and biochemical characteristics of Monilia psilosis which are used to decide the species, have in my work been found true also in some respects of wild yeasts, and yeasts from blastomycosis. When such characteristics are common to other species they lose their value as diagnostic aids. Among numerous writers who have been unable to identify Monilia psilosis by cultural methods as an organism specific for sprue are Manson-Bahr,[5] Dold [3] and Bastedo and Famulener.[6] Castellani [4] does not believe the species monilia can be distinguished on purely morphologic and botanical characters, but that immune phenomena must be considered also. Ashford working with a large number of strains and many varieties of sugar mediums appreciates that the biochemical methods alone are not sufficient and, at his instigation, Michel has carried out complement-fixation reactions which quite definitely indicate a pathogenic rôle for the yeasts, present in stools and mouth lesions of sprue patients. It might be questioned whether this indicates a definite species because the controls did not include strains of other forms of pathogenic yeasts, such as those found in blastomycosis.

SUMMARY

The morphologic and biochemical reactions of old strains of Monilia psilosis are not sufficiently distinctive to differentiate them from other closely related nonpathogenic fungi.

Common agglutinins for yeasts from various sources are produced readily in rabbits by injecting strains of Monilia psilosis and blastomycetes.

[4] Manual of Trop. Med., 1913.
[5] Manson's Tropical Diseases, 1921.
[6] Jour. Am. Med. Assn., 1923, 81, p. 2101.

Mycotic abscesses were constantly produced in animals by injecting old cultures of Monilia psilosis and were not produced by wild yeasts or yeasts from blastomycosis.

The failure of sprue yeasts and blastomycetes to absorb completely agglutinins for the opposite strain might indicate a degree of specificity, but the evidence as a whole does not point to Monilia psilosis as a well defined species.

EXPERIMENTAL STUDIES ON THE BACTERIOPHAGE

TWO PLATES

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D'Herelle's [1] observations and his "vital" theory appeared too inconsistent with the laws of nature to many bacteriologists. The experiments reported here were made in the hope of throwing some light on the cause of the bacteriophage phenomenon.

TECHNIC

This new field of investigation brings with it new methods of research, and for this reason I have thought it necessary to give some of the details of the technic used in my work.

Broth.—The reaction of broth is adjusted to P_H 8.0, and 0.5% of sodium chloride is added. If it is necessary to observe a change in reaction after inoculation, 1 cc. of a 0.01% solution of phenol red is added. Control experiments have shown that this quantity does not interfere with the growth of bacteria.

Agar.—The reaction of the stock broth is adjusted to P_H 7.0-7.4, 3% agar and 0.5% sodium chloride is added.

Filters.—Throughout this work the Chamberland L 2 and L 3 filter candles were used.

Isolation of Different Strains of Bacteriophage.—If a particular bacteriophage is wanted, isolation is facilitated by an enrichment process. This consists of adding a few drops of an emulsion of the bacteria against which we wish to obtain a bacteriophage to the mixture of feces in broth. The bacteriophage, if present, will multiply during the first incubation and will be more easily detected.

Technic of Passages.—Greater attention was paid to the phenomenon of clearings than to the lysis of the emulsion, since every change in activity or virulence of the bacteriophage at once changed the aspect of the clearings. About 0.02 c c. of a filtrate was added to 10 c c. of a

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[1] Compt. rend. Acad. d. sc., 1917, 165, p. 373; Le Bacteriophage, 1921.

bacterial suspension and one loopful (0.02 c c.) transferred to another 10 c c. of bacterial emulsion. A loopful of the last dilution was spread over a quarter of a Petri dish.

Lysis.—Every strain of bacteriophage has a different mode of lysis, and further may show wide alteration in its lytic properties. The completeness of lysis is quite independent of its rapidity; sometimes lysis is almost complete in 2 hours, with a slight turbidity which may persist for many hours or never disappear. Lysis may come about suddenly; an emulsion at the third hour of incubation with a turbidity equivalent to 350 mm. bacteria per c c. may in about 5 minutes become quite limpid. On the other hand, lysis, commencing early, goes on gradually without any sharp point at which it begins, while with other strains lysis progresses so slowly that only after 48 hours will there be complete lysis.

"Clearings."—The "clearings" present a wide variation, not only between different strains (figs. 1, 2, 3, 4 and 5), but also between one and the same strain at different times, due to known or unknown causes (figs. 5, 8, 9, 1 and 4). Ordinarily the dimensions of the clearings are in proportion to rapidity of lysis—the greater the clearings the quicker is lysis. However, this is not invariably so, for we have observed an anti-shiga bacteriophage which gave clearings from 1 to 2 mm. in diameter (fig. 4), but which failed to clear an emulsion. Bacteriophage strains with slow action give clearings with sharply cut outlines (fig. 1), which grow slowly and seldom attain a diameter of more than 2 mm., while strains with quick action give larger clearings, from 12 to 15 mm. in diameter (fig. 5). However, ordinarily their outlines are not sharp, since they are surrounded by a gradually shelving layer of bacteria, forming a "zone of corrosion" (fig. 7). The growth of these large clearings is easily followed. When, after 2½ to 3 hours' incubation, the bacterial growth is appreciable, the clearings are already marked as clear areas from 2 to 3 mm. in diameter. After 6 hours they attain a diameter of from 3 to 4 mm., and it is during this time that the "zone of corrosion" appears. After 24 hours the clearing is still sharply enough outlined (fig. 10), and though the contours begin to lose their sharpness, it is still possible to follow their enlargement, until, after from 48 to 72 hours, the whole bacterial layer between the clearings is included in the "zone of corrosion" (fig. 11).

Secondary Growth of Bacteria.—With some strains of bacteriophage, on the bare area of the clearings small colonies of bacteria appear, so-called secondary growths. Small clearings, produced by a slowly

acting but virulent strain of bacteriophage, are never, in our experience, covered with such colonies. On the other hand, with a quickly acting but less virulent strain these colonies appear, sometimes, after from 6 to 8 hours' incubation. It must be pointed out that secondary growth appears only on a completely bare area and never in the "zone of corrosion," where some of the normal growth remains (fig. 11). The dimensions of the secondary colonies appear to depend on the area which is free from normal growth.

The number of secondary colonies on agar bears no quantitative relation to the completeness of lysis in the broth from which the smear was made. For, if after inoculation of an emulsion with the bacteriophage, we smear a part of it on agar, we may obtain many secondary colonies and a complete lysis in broth without secondary growth, or vice versa.

Study of the Variability in the Properties of the Bacteriophage.—Having become acquainted with the technic, we chose a strain of bacteriophage for closer study. The strain chosen, "B. 5. FL. II" active against B. dysenteriae, Flexner, strain 2, was isolated by the enrichment method, from the feces of a healthy guinea-pig. It was not very active at first, but it reached such a degree of activity after a few passages that it became our "favorite" strain, in spite of its instability in virulence. At times it showed great activity, 6,000,000 ultramicrobes dissolving 10 c c. of a normal emulsion of bacteria in 1 hour and 45 minutes; at other times it would become very slow, the same number of ultramicrobes only clarifying but never dissolving the emulsion. The resistance of this strain to heat was also subject to variations, sometimes resisting 70 C. and at other times only 65 C. for 5 minutes.

The clearings on agar were characteristic (figs. 5, 7 and 9). At a time of greatest activity, after 24 hours' incubation, some of the clearings measured not less than 15 mm. in diameter, including their "zone of corrosion," the complete bare area in the center measuring 5 mm. across (fig. 5). At the same time there were clearings present measuring only from 1 to 3 mm. in diameter and of the same characteristic appearance. At times of diminished activity the clearings were smaller, and the "zone of corrosion" did not widen so rapidly.

Lysis was seldom absolute, even though ideal limpidity of the emulsion took place; in a great majority of cases, after some days, a slight sediment of resistant bacteria appeared at the bottom of the tube. Secondary colonies in the bare area of the clearings were almost always

obtained, the number and rapidity of their growth depending on the state of activity of the bacteriophage.

This strain of bacteriophage was also active against other strains of Flexner, but with a lower virulence, and also against a few strains of B. typhosus, B. coli and B. dysenteriae shiga. After many months of daily passage this virulence was lost and only Flexner emulsions were affected.

When kept in ordinary tubes in a dark place, either in the form of filtrate or unfiltered lysates, this strain loses its activity in from 3 to 5 months, and even after 2 months it was found to be considerably attenuated. I regard the following experiment of the greatest importance in support of the theory of the nature of the bacteriophage.

A filtrate of this strain, containing 200,000,000 bacteriophages and giving clearings with a diameter of 4.5 mm., after two months was inoculated into a normal culture of bacteria. The first tube, receiving 0.003 c c., the second 0.02 c c. and the third 3 c c.; immediately after inoculation 0.02 c c. of each mixture was transferred to agar. The following results were observed (fig. 12):

First Mixture: There were about 140 clearings of medium size, which after from 4 to 5 hours' incubation began to be covered with minute secondary colonies. After 24 hours, clearings were seen lying between normal layers of bacteria. They were detected only because the secondary colonies did not become confluent and appeared as bluish spots on a whitish ground. In the tube lysis was just perceivable.

Second Mixture: There were many confluent "traces" of clearings, with a few areas of normal bacterial growth. One large clearing was completely free from secondary growth. In the tube there was a slight degree of lysis.

Third Mixture. There were no signs of normal bacterial growth. The whole surface was covered with the characteristic colonies of secondary growth (fig. 12 C), and on this background were many clearings completely free from any bacterial growth (fig. 12 D). In the tube lysis was considerable only after 4 hours.

From this experiment the following conclusions are drawn: After 2 months' storage only about 1 in 300 of all bacteriophages present survived. Only a few, about 0.5%, of those that survived maintained their virulence. During subsequent passages the number of "avirulent" clearings diminished, and lysis gradually attained its normal time. The results were not due to a change in the resistance of the bacteria, since

after the series of passages had been made, we ran parallel tests with the old attenuated filtrate and the recovered filtrate, using emulsions prepared from the same agar slant, and obtained results identical with those recorded above.

This experiment may well be taken as an illustration of the vital properties of the bacteriophage and may serve as an answer to Bordet's [2] demand for the proof of the living nature of the virus, as stated by him in the following terms.

> One can hardly believe a virus to be always ready to attack the bacterium, however long may be the lapse of time during which it has been starved—that is has not been supplied with the living bacteria representing its necessary food. When kept inactive during a long period, the virus in all probability should require an appreciable delay before eliciting its normal aggressive abilities.

As was stated, the virulence of this strain of bacteriophage was subject to great changes in spite of daily passages. Although the routine technic was unaltered from day to day, we searched for an external cause of the periodic loss in virulence, finally coming to the conclusion that the quality of the medium exerted some influence. We observed that with B. dysenteriae Flexner an acid reaction rather rapidly developed in our broth, and with some batches of broth the reaction change still more rapidly, particularly during the first hours of incubation, although no traces of sugar were found by Bertraud's method.

TABLE 1

TWO HUNDRED AND FIFTY MILLIONS OF FLEXNER "II" IN PLAIN BROTH

Interval	0 Hours	2 Hours	3 Hours	5 Hours	8 Hours	10 Hours	24 Hours
P_H value	8.0	7.8	7.4	7.2	7.0	7.0	6.6

D'Herelle's experience has shown that minimal traces of acid were poisonous to the bacteriophage, and believing that the daily action of acid attentuated the virulence of our bacteriophage, we tried to stabilize the reaction of our medium. We added insoluble salts of magnesium to ordinary peptone water in which the quantity of "buffers" had been augmented by our concentrated broth, and finally reached a quite unexpected result, namely, that the more stable the reaction the slower lysis took place.

This led us to try the effect of increasing the rapidity of change in reaction by the addition of 1% of glucose to the broth. A directed read-

[2] Brit. Med. Jour., 1923, p. 175.

ing of P_H was made by adding 1 c c. of a 0.01% solution of phenol red to the tubes.

It will be seen that in ordinary broth lysis commenced after 3 hours and approached completion only after 7 hours, whereas in the glucose broth, in spite of an increasing acidity, lysis began within 2 hours and in an interval of from 40 to 45 minutes gave an ideally limpid fluid. The acidity of the glucose broth after 24 hours and its turbidity were due to a secondary growth of bacteria, but if, as happened occasionally, all bacteria were destroyed, no such secondary turbidity occurred.

TABLE 2

RESULT ON ADDITION OF GLUCOSE

	Initial		2 Hours		2 Hours 30 Min.		2 Hours 35 Min.		3 Hours		4 Hours		7 Hours		24 Hours	
	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T
Ordinary broth	8.0	200	7.5	250	7.5	250	7.3	300	7.3	300	7.3	100	7.3	40	7.31	100
Broth with 1% of glucose....	8.0	200	7.0	100	7.0	25	7.0	0	7.0	0	7.0	10	7.0	15	6.6	500

TABLE 3

EMULSION OF B. TYPHOSUS "K" INOCULATED WITH BACTERIOPHAGE 5

	Initial P_H	Initial T	T after 2 Hours 30 Min.	T after 3 Hours 30 Min.	T after 5 Hours 30 Min.	T after 24 Hours
Ordinary broth.....	8.0	250	350	350	300	100
1% glucose broth.....	8.0	250	400	100	100	500

It seemed interesting to determine whether this peculiarity of the strain of bacteriophage used, to act better in an acidifying medium, was an inherent property of the bacteriophage or of the bacterium. Although I subjected other strains of bacteriophage, antityphosus, antishiga, and others, to the same treatment, all failed to show any lysis in a medium with increasing acidity. Having at this time no other strain of bacteriophage active against B. dysenteriae Flexner 2, I took advantage of the virulence which strain "B 5-Fl. II" showed for other bacteria against which I had strains of bacteriophage. Although this virulence was slight, I could quite clearly see that lysis was affected more quickly with "B 5-Fl. II" in an acidifying medium than in a normal medium. A strain of bacteriophage active for the same B. typhosus "K" failed to produce any lysis under such conditions, acting only in ordinary alka-

line broth. The same phenomena were observed B. dysenteriae Flexner "2015" and with a bacteriophage active for this culture. From this, then, it is clear that the type of lysis depends only on the bacteriophage and not on the bacterium.

The next step was to search for the cause of this difference between strains of bacteriophage. There is evidence in the literature that the bacteriophage can be gradually accustomed to live and act under conditions which would normally destroy it. D'Herelle [3] showed that it was possible to accustom the bacteriophage to the action of glycerol, Prausnitz,[4] and recently Bruynoghe and Wagemans,[5] to the action of specific antiserum, Wolf and Jansen,[6] to the action of chinosol. Thinking that strain "B 5. Fl.II" had become accustomed to acid during many passages through an acidifying medium, I proceeded to accustom another bacteriophage to the action of acid by passages through broth of higher initial acidity. For this purpose our bacteriophage strain "2015" active for B. dysenteriae Flexner "2015" was chosen.

TABLE 4

EMULSION OF B. DYSENTERIAE FLEXNER "2015" INOCULATED WITH BACTERIOPHAGE "2015"

	Initial		1 Hour 30 Min.		3 Hours 30 Min.		4 Hours 30 Min.		5 Hours 30 Min.		8 Hours		24 Hours	
	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T
Ordinary broth	8.0	250	7.5	250	7.3	200	7.3	75	7.5	50	7.3	25	7.3	25
Ordinary broth	7.0	250	6.8	300	6.6	300	6.6	300	6.6	250	6.6	200	6.6	100
1% glucose broth	8.0	250	7.4	400	6.6	500	6.6	500	6.6	500	6.6	500	6.6	500

From this table it is seen that although the bacteriophage gave an active lysis in the broth with initial P_H 8, it acted feebly in broth with a P_H of 7 and failed to act in an acidifying medium.

I then proceeded to pass strain B 2015 first through neutral broth and later through broth of an initial acidity. As a control, the same strain was also passed through alkaline broth, and the two were called "B 2015 acid" and "B 2015 alk," respectively. The progress of the experiment is clearly shown in table 5.

After the eleventh passage in broth with an initial acidity of P_H 6.6, its activity was tested in glucose broth, controlling the results as is shown in table 6.

[3] Le Bacteriophage, 1921, p. 92.
[4] Centralbl. f. Bakteriol., I, 1922, 89, p. 187.
[5] Compt. rend. Soc. de biol., 1923, 88, p. 968.
[6] Ibid., 1922, 87, p. 1087.

From this experiment it is clear that strain B. 2015 after 12 passages in neutral and acid broth acquired a resistance to acid, as was shown by its ability to lyse in an acidifying medium. Neither of the control strains B. 2015 Alk. or the original strain of B. 2015 showed this ability

TABLE 5

RESULT OF PASSING STRAIN B 2015 THROUGH NEUTRAL BROTH AND THROUGH BROTH OF AN INITIAL ACIDITY

	Initial Turbidity	Turbidity after 2 Hours 30 Min.	Turbidity after 3 Hours 3 Min.	Turbidity after 4 Hours 30 Min.	Turbidity after 5 Hours 30 Min.	Turbidity after 8 Hours 3 Min.	Turbidity after 24 Hours
		Second	passage	in neutral	medium		
B. 2015 acid in ordinary broth of initial P_H 7.0	300	400	450	450	450	350	150
B. 2015 alk. in ordinary broth of initial P_H 8.0	300	400	350	250	150	100	100
		Fifth	passage	in neutral	medium		
B. 2015 acid in ordinary broth of initial P_H 7.0	250	350	300	250	200	150	50
B. 2015 alk. in ordinary broth of initial P_H 8.0	250	350	400	350	250	200	75
		Eighth	passage,	acid	medium		
B. 2015 acid in ordinary broth of initial P_H 6.6	300	300	300	200	200	200	25
B. 2015 alk. in ordinary broth of initial P_H 8.0	300	250	250	150	150	100	25

TABLE 6

TWELFTH PASSAGE OF BACTERIOPHAGE B. 2015

	Initial		2 Hours		2 Hours 30 Min.		3 Hours		4 Hours		6 Hours		24 Hours	
	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T	P_H	T
1. "B. 2015 Acid" in 1% glucose broth	8.0	250	6.8	450	6.6	75	6.6	10	6.6	10	6.6	25	6.6	500
Controls:														
2. "B. 2015 Alkline" in 1% glucose broth	8.0	250	7.3	400	7.1	500	7.1	450	7.1	400	6.8	500	6.6	500
3. B. 2015 Original filtrate in 1% glucose broth	8.0	250	7.3	450	7.0	500	6.6	500	6.6	500	6.6	500	6.6	500

Since the bacterial suspension was prepared from the same agar slan there can be no question of a change in the bacteria.

It may also be stated that the strain which had been passed through an acid broth partly lost its virulence. This is explained on the basis of a more rapid production of acid in tube 1, table 6, than in the other

tubes, due to the fact that the bacteria were better able to exercise the vital function than in the presence of alkali tolerant bacteriophages.

These alkali tolerant strains quickly lose their activity as the acidity of the medium increases.

STUDY OF THE NATURE OF CLEARINGS

Two explanations of the nature of the clearings have been given, that of d'Herelle, who sees in them the colonies of the bacteriophage, while the opponents of this view regard them as the places where the bacteria most sensitive to the "lytic principle" are located. If the latter explanation is accepted, it must be supposed that the nonvital "lytic principle" is a substance dissolved in bacteriophagic fluid. The activity of this fluid must then depend on the concentration of this substance. On this basis Gratia and De Kruif [7] and others determine the activity of such a fluid by the dilution method, stating the highest dilution in which the fluid still shows activity. It must then be assumed that the less the concentration, the feebler will be the action, and the fewer the number of sensitive bacteria attacked at the outset. Experimentation does not reveal any such dependent relation, as is shown by the following.

Using Wright's technic, I placed on a paraffined slide two equal volumes of bacteriophage diluted so as to give only a few clearings. To the first volume was added 1 volume of bacterial emulsion, and to the second, 9 volumes. After mixing thoroughly the 2 dilutions in their entirety were spread on agar, and 135 clearings developed from the first dilution and 117 from the second. Since I was dealing with such a small number of bacteriophages, I was in the zone of "errors of chance" (Wright). I used other methods of measuring the liquids: measuring with a small loop the dilution of 1:1 gave 15 clearings; the 1:9 gave 21 clearings (fig. 5). Measuring a big loop: dilution 1:1 gave 49 clearings; dilution 1:9 gave 65 clearings. Measuring by the drop method and mixing directly on the agar surface gave 46 clearings from the 1:1 dilution and 47 clearings from the 1:9; although the concentration of the first mixture was 5 times greater than that of the second and although in the second there were 9 times as many bacteria among which the "lytic principle" must have been divided, the number of clearings obtained was practically the same. Apparently neither the concentration nor the number of bacteria has any influence on the number of clearings which result.

[7] Ibid., 1923, 88, p. 308.

Following the theory of a soluble substance, I must admit that only the total quantity of the substance present is of importance. The quantity of liquid giving 10 clearings contains a quantity of "principle" just sufficient to dissolve 10 of the most sensitive bacteria. In this case the phenomenon of absorption of the total quantity of "principle" must be accepted. Bordet [2] expresses this in the following terms: "clear spots appear only at the spots where such (sensitive) bacteria have been deposited and only when they have absorbed a sufficient quantity."

To control this supposition, I attempted to diminish or increase the possibility of absorption by taking a small volume of diluted bacteriophage, spreading it over an agar surface, allowing it to dry, and then covering this area with a suspension of bacteria. In this way the absorption was restricted to the area immediately surrounding the bacterium.

Experiment: To 10 c c. of broth was added a small quantity of bacteriophage. Two hundredths c c. of this mixture was spread on agar and the dish placed in the incubator until the drop had completely dried. I prepared a bacterial emulsion and spread a large loopful over the prepared surface. As a control I added the same quantity of bacteriophage to 10 c c. of a bacterial emulsion, and after 30 minutes at room temperature spread 0.2 c c. of this mixture on agar. On the prepared plate, 35 clearings were obtained; on the control, 31 clearings.

It may be argued, however, that beyond the clearings there may be some "principle" left and that the scarcity of sensitive bacteria prevented its detection. The following experiment proves the contrary:

On 4 equal agar surfaces I spread equal quantities of bacteriophagic liquid, and when the surfaces were completely dry, I smeared on each area bacterial emulsions of different density. Surface covered with emulsion of 2,000,000,000 bacteria per c c. shows 21 clearings; surface covered with emulsion of 400,000,000 bacteria per c c. shows 24 clearings; surface covered with emulsion of 80,000,000 bacteria per c c. shows 26 clearings; surface covered with emulsion of 16,000,000 bacteria per c c. shows 17 clearings.

Trying to diminish still more the possibility of absorption, I made the following experiments:

From 10 c c. of broth containing a diluted bacteriophage we spread 0.02 c c. on the surface of a sterile slide, and when the slide was completely dry it was placed face down on a surface of agar previously inoculated with bacterial emulsion. Control: To the same tube of liquid from which I prepared the slide I added bacteria, and after spreading

0.02 c c. of this on agar, I covered the surface with a sterile slide. Under the first slide I obtained 13 clearings; under the control, 10 clearings.

Two surfaces of agar were covered with equal quantities of bacteriophage liquid, and when the surfaces were dry I covered one with bacterial emulsion as gently as possible, and in covering the other I used friction, rubbing to and fro as much as possible. On the first I obtained 49 clearings, and 36 on the second. Another experiment gave 35 and 32 clearings, respectively.

A further experiment confirms these results. On the surface of an agar plate 2 areas were marked, one of 16 sq. cm., the other of 4 sq. cm., and equal quantities of bacteriophage liquid were spread on them. In this way I had different concentrations of "principle," and in the case of the small square I might expect that the less sensitive bacteria would also be affected, resulting in a greater number of clearings. This was not the case, however, since the large square gave 13 clearings and the small square 11 clearings.

Further acquaintance with different types of clearings of the bacteriophage has led ot interesting observations. As d'Herelle stated, every strain of bacteriophage has peculiarities which differentiate it from others. Bail and Watanabel [8] have shown that it is possible to separate a bacteriophage into several types and to cultivate these types. On the other hand, Gratia and De Kruif [9] deny this possibility. I have been able to separate two different strains of bacteriophage showing different types of clearings, after having mixed them.

DISCUSSION

The research described in the preceding pages has left the impression that the bacteriophage must be accepted as an ultramicrobe, an obligatory parasite of bacteria.

In practically all publications on the phenomenon of transmissible lysis, of which the greater part are against the vital theory, we find facts characteristic, not of bacteria, which undergo lysis, but of one or another "sample" or strain of bacteriophage. D'Herelle [1] says clearly that every strain has its own peculiarity. Cruz,[10] although admitting the ferment theory, speaks of differences between various samples of lytic principle acting on the same bacterium. Bruynoghe and Brut-

[8] Wien. klin. Wchnschr., 1922, 35, p. 8.
[9] Compt. rend. Soc. de biol., 1923, 88, p. 629.
[10] Ref. Bull. Inst. Pasteur, 1922, p. 955.

saert,[11] Applemans and Wagemans,[12] Bruynoghe and Appelmans,[13] Tchang Kono Ngen and Wagemans[14] and Handuroy[15] clearly show the differences between different strains of bacteriophage acting on the same strain of bacteria. I have shown that different strains acting on the same bacterium can be distinguished by their peculiarities, even going so far as to mix them and again isolate them in a pure state. Is it logical to state that the same bacterium can produce an unlimited number of different autolytic principles? If we accept the existence of 2, 3 or more, we have no right to deny the existence of an unlimited number. It has been proved that these manifestations of variability are peculiar to the bacteriophage, not to the bacterium attacked, and variability is peculiar to living substance.

In accord with the observations of other investigators, it has been shown possible to accustom a strain of bacteriophage to the action of acidity, and proof has been given that it is the bacteriophage that acquires this property and not the lysed bacteria. "L'accoutumance est l'apanage exclusif des êtres vivants," d'Herelle.

The concentration of a liquid in "principle" has no influence on the number of clearings, nor has the number of sensitive bacteria any influence. There is no "principle" left outside of that in the clearings. Although the total quantity of "principle" is in action, there is no preliminary absorptoin of it by sensitive bacteria (fig. 9). There is no substance dissolved in bacteriophagic liquid. We are dealing with numbers of "something," not quantity, that is, there are present some enumerable bodies, independent of bacteria, which give rise to the clearings.

All these data lead me to accept the existence of Bacteriophagium intestinale (d'Herelle, 1917), admitting only one other eventual possibility, namely, that the phenomenon is possibly a new manifestation of life, not comparable with any other manifestation known. In the present state of knowledge, d'Herelle's phenomenon cannot be explained logically other than by the presence of a living being.

SUMMARY

There are no theoretical proofs against the existence of the bacteriophage.

[11] Compt. rend. Soc. de biol., 1923, 88, p. 966.
[12] Ibid., 1922, 86, p. 738.
[13] Ibid., 1922, 87, p. 96.
[14] Ibid., p. 1253; 1923, 88, p. 393.
[15] Ibid., 1922, 87, p. 1089.

There is a great variability in the manifestations of different strains of bacteriophage even against the same bacterium.

A given strain is subject to wide variations in its activity.

The virulence of a strain of bacteriophage becomes attenuated rapidly, but can be regained by passages.

One strain of bacteriophage had the property of acting in a medium of increasing acidity. This was shown to be a peculiarity of the bacteriophage and not of the bacterium.

It is possible to accustom the bacteriophage to act under conditions which normally render it inactive.

The concentration of a liquid in "lytic principle" has no influence on the number of clearings. There is no absorption of a hypothetical "lytic principle" by sensitive bacteria. There is no dissolved substance present. There are some enumerable bodies present.

It is possible, after mixing two different strains of bacteriophage, to separate them again in the pure state.

All the observed facts prove the existence of a living being, Bacteriophagium intestinale (d'Herelle, 1917).

PLATE 1

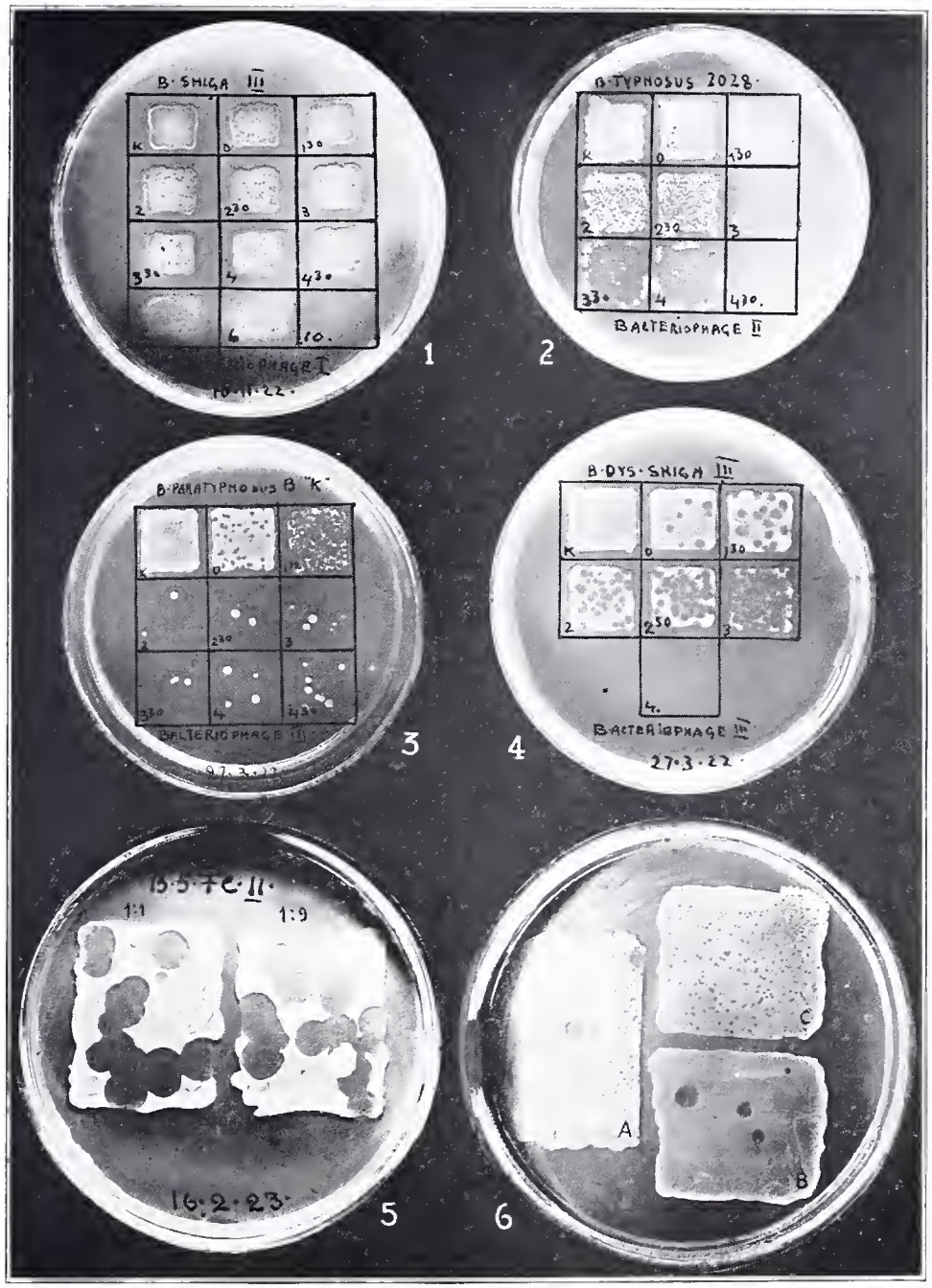

Fig. 1.—Antishiga bacteriophage 1. In this and the following illustrations, *K* indicates control, emulsion before addition of bacteriophage; *O*, the same emulsion immediately after addition of bacteriophage. The other numbers indicate the time of incubation after which the transfers on agar were made.

Fig. 2.—Antityphosus bacteriophage 2. Note the curious secondary growth protruding outside the squares inoculated.

Fig. 3.—Antishiga bacteriophage 3. Note the different type of clearings produced by other bacteriophage—the I one (fig. 1) with the same strain of bacteria—shiga B (see p. 2).

Fig. 4.—Bacteriophage 3. The same as on fig. 3, but acting on B. paratyphosus B.; quick acting, but a virulent strain of bacteriophage.

Fig. 5.—"B-5-Fe-II," bacteriophage 5 acting on B. dysenteriae Flexner 2 in its active period. This figure illustrates the experiment mentioned on p. 537.

Fig. 6.—"B-5-Fe-II" and "B 466" mixed (*A*) and separated: *B*, "B 5 Fe II"; *C*, "B 466."

TUBERCULOSIS OF MAMMALIAN TISSUE IN VITRO

SIX PLATES

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INTRODUCTION

The interrelations between tissue elements and pathogenic microorganisms constitute a problem, which can be successfully attacked with the aid of tissue culture methods. The behavior of cells toward bacteria in a tissue culture can be easily observed in living condition and followed step by step in its successive stages. In this paper I communicate the results of my investigations on the simultaneous cultivation of mammalian tissues and tubercle bacilli in vitro. The object of my research was purely morphologic—I tried to reproduce in vitro, if possible, the characteristic pathologic picture of the tuberculous process, as it is found in the infected organism. I thought, that if this aim could be attained, the histogenesis of the typical tuberculous formation, the tubercle, still doubtful in many respects, might easily be elucidated.

The tuberculous process in the living organism may be looked on as a specific inflammation with characteristic anatomic and histologic features, with rapid or slow progress. The presence of tubercle bacilli in a tissue calls forth the formation of more or less numerous peculiar nodules, the so-called tubercles; in growing and agglomerating, they become visible to the naked eye, and finally may form large tumor-like foci. Their ultimate fate is a special type of degeneration—the caseous degeneration or caseation. If the process takes a turn toward healing, the caseous tubercle may be penetrated and resorbed by the surrounding connective tissue, forming in its place a scar, or it may become surrounded by a dense connective tissue capsule and sometimes even calcify.

The chief constituent part of a tubercle are the so-called epithelioid cells—large, mostly polyhedral elements with abundant protoplasm, usually containing a varying number of tubercle bacilli, a large nucleus and a highly developed cytocentrum with numerous centrioles and a large attraction sphere (Wallgren,[1] Castrén[2]). They are arranged in

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[1] Arb. a. d. path. Inst. d. Univ. Helsingfors, 1911, 3, p. 139.
[2] Ibid., 1923, p. 1.

an epithelium-like fashion, without an appreciable quantity of intercellular substance between them. The second almost constant element of a tubercle are the large multinucleated giant cells of Langhans, usually also containing bacilli. The peripheral arrangement of their numerous nuclei and the pale, often homogeneous appearance of the central area of the cell body with a large cytocentrum (Wakabayashi [3]) are considered as typical. Finally, the third element are lymphocytes and, sometimes, special, granular, polymorphonuclear leukocytes, confined, as a rule, to the outer zone of the tubercle. Blood vessels are either totally absent, or are found only at the periphery, in the form of a few capillaries. It is self-evident that common fibroblasts are also always present in the connective tissue surrounding a tubercle. Between the different cells of the latter, remnants of various degenerated parts of the original tissue may be present. Although tubercle bacilli, as a rule, are present in the cells of the tubercle, they may be scarce or totally absent in some cases.

Despite the extensive literature, the question of the origin of the various elements of the tubercle can by no means be considered as settled; new papers dealing with this problem continue to appear. I need not give a complete bibliographic list of the literature concerning the histogenesis of the tuberculous lesions. Exhausting references may be found, especially in the papers of Wallgren,[1] von Fieandt [4] and in the recently published article of Castrén.[2]

Regarding the origin of the most typical elements of the tubercle, the epithelioid cells, the opinions of the authors were, from the very beginning, divided in two principal groups that have remained up to the present time. Whereas Cohnheim and R. Koch, and, following them, the French school, Metschnikoff,[5] Borrel [6] and others, claimed for the epithelioid cells an origin exclusively from blood leukocytes, German investigators, such as Baumgarten,[7] Orth [8] and others, traced them back exclusively to the fixed, local elements of the tissue.

More recently the possibility of the development of epithelioid cells from the white blood cells, i. e., from their nongranulated variety, the lymphocytes, has been supported by a series of investigators, using

[3] Virchows Arch. f. path. Anat., 1911, 204, p. 421.
[4] Arb. a. d. path. Inst. Helsinfors, 1911, 3, p. 235.
[5] Virchows. Arch. f. path. Anat., 1888, 113, p. 63.
[6] Ann. de l'Inst. Pasteur, 1893, 7, p. 593.
[7] Verhandl. d. deutsch. path. Gesellsch., 1902, 4, p. 2.
[8] Ibid., p. 30.

experimentally produced tuberculosis in animals—Buday,[9] in tuberculosis of the kidney, v. Fieandt in the meninges and the brain, Homén [10] in the peripheral nerve trunks and chiefly Wallgren in the liver. Confirming the results obtained by Maximow [11] for the experimental aseptic inflammation, namely, the development of the mononuclear ameboid inflammatory cells, the polyblasts, partly from emigrated blood lymphocytes, partly from mobilized local resting wandering cells, the investigators just mentioned found also for the tuberculous inflammation the same development of polyblasts partly from fixed elements and partly from lymphoid blood cells. These polyblasts engulfed the tubercle bacilli, hypertrophied and transformed themselves into the typical epithelioid elements of the tubercle. Maximow, in his monograph of 1902, had also emphasized that the epithelioid cells of the tubercle are merely a special type of polyblasts, and, as such, evidently have to originate partly from hematogenous lymphoid cells.

But this idea of the development of the polyblasts and, in the special case of tuberculous inflammation, of the epithelioid cells of the tubercle from hematogenous lymphocytes did not become popular. There is a widespread tendency at the present time among the various authors to ascribe the formation of the elements of the tubercle exclusively to local fixed cells. In view of the advanced knowledge of the normal histology of the connective tissue, these fixed cells nowadays, however, are not called indiscriminately "fixed connective tissue cells," as in the days of Baumgarten,[7] but various special types are distinguished; among them the fibroblasts, the endothelium of blood vessels and especially the so-called histiocytes of Kiyono [12] have to be mentioned. The partisans of the exclusive fixed cell theory either ascribe to all of these different fixed cell types and even to the epithelial cells the capacity of forming tubercles, as for instance Lubarsch [13] in the Textbook of Pathology of Aschoff (p. 561), or they show a predilection toward some special types of them. Thus, Castrén [2] in a recent paper emphasizes that in tuberculosis of human connective tissue the epithelioid cells originate chiefly, if not exclusively, from fibroblasts. Foot [14] is a partisan of the exclusive endothelial theory. Goldmann,[15]

[9] Virchows Arch. f. path. Anat., 1906, 186, p. 145.

[10] Arb. a. d. path. Inst. d. Univ. Helsingfors, 1922, 3.

[11] Beitr. z. path. Anat. u. z. allg. Path., 1902; Supp. 5, 1903, 34, p. 153; 1903, 35, p. 93; 1905, 38, p. 301.

[12] Die vitale Karminspeicherung, 1914.

[13] Aschoff, L.: Pathologische Anatomie, 1923, 1, p. 514.

[14] Jour. Exper. Med., 1920, 32, p. 513, 539; 1921, 33, p. 271.

[15] Beitr. z. klin. Chir., 1909, 64, p. 192.

Joest and Emshoff,[16] Kiyono [12] and Evans, Bowman and Winternitz,[17] using vitally stained animals for experimental tuberculosis in various organs, showed that the so-called histiocytes—the cells of Kupffer in the liver, the reticular cells in the lymphnodes, etc.—are responsible for the production of epithelioid cells, although Kiyono and Evans, Bowman and Winternitz could not absolutely exclude the possibility of a participation of blood cells of polyblastic type.

The confusion concerning the endothelium and the histiocytes is chiefly due to the instability of the ideas of the true nature of these cells.

That the giant cells of Langhans originate from the epithelioid cells of the tubercle, is an established fact. The unsettled question is merely the mode of their formation. Usually amitotic, in some cases also mitotic division of the nuclei in the hypertrophying cell body is made responsible. The possibility of fusion of single epithelioid cells, although somewhat reluctantly admitted by some writers (Joest and Emshoff,[16] Lubarsch,[13]) seems to be in general unpopular.

Regarding the lymphocytes of the tubercle, the question of their origin is subjected to the same controversies as in the common aseptic inflammation, and whereas some investigators insist in sharply distinguishing hematogenous and histiogenous lymphocytes, and claim for the lymphocytes of the tubercle the one or the other origin, the most natural point of view certainly is the complete abolition of any distinction between lymphocytes of various origin, as emphasized on various occasions by Maximow.[18] According to what has been said previously, the lymphocytes of the tubercle, of whatever origin they may be, are by most authors not believed to play an important rôle in the production of epithelioid and giant cells.

The special granular leukocytes, if they occur in the tubercle, evidently always come from the blood vessels.

It is difficult to attempt a solution of all the enumerated questions on human material, where usually only old stages of the process are available. However, in the experimental material many difficulties are evidently encountered as well; we have seen that in the hands of the various experimenters it has also furnished contradictory results.

The advantage of the method of tissue cultures for studying the interrelations between the tubercle bacilli and the tissue cells, from the morphologic as well as from other points of view, is obvious. All

[16] Virchows Arch. f. path. Anat., 1912, 210, p. 188.

[17] Jour. Exper. Med., 1914, 19, p. 283.

[18] Arch. f. mikrosk. Anat., 1909, 73, p. 444; Verh. d. Internat. Congress z. Budapest, 1909; Folia haematologica, 1909, 8, p. 125.

the complicating influences of the other elements of the body and especially the interference of hematogenous cells can be avoided and, besides, the changes in the tissue can be easily watched directly in the living condition.

Experiments on the behavior of living cells of the vertebrate organism, when in contact with micro-organisms outside the body, have been performed by several investigators (Lewis,[19] H. F. Smyth,[20] and others). The publications most important for our present problem are the papers of Smyth and D. T. Smith, H. S. Willis and M. R. Lewis.[21]

H. F. Smyth cultivated human tubercle bacilli with chick embryo tissues in chicken plasma and observed phagocytosis of the bacilli by connective tissue and even epithelial cells. He did not undertake special histogenetic studies of this process in vitro, but he made several extremely interesting statements, which will be fully confirmed in this paper. He saw a clustering of small round cells, interpreted as lymphocytes, around bacillary clumps and colonies, followed by a similar clustering of larger "parenchymatous" polygonal cells with one or several nuclei, interpreted as epithelioid cells. They phagocytized the bacilli with which they came in contact. The larger cells, containing bacilli, then fused to form multinucleated giant cells with nuclei in characteristic groupings and bacilli in degenerated protoplasm away from the nuclear groups.

Smith, Willis and M. R. Lewis [21] confirmed some of the findings of Smyth.[20] They used cultures of chick embryo tissue in the artificial Lewis medium and inoculated them with avian tubercle bacilli. They could not find, however, any growth of the bacilli in the medium used, and observed only an accidental engulfing of single bacilli by various cells, the phenomenon being, in their opinion, a purely physical one and of precisely the same character as when the cells meet other foreign bodies. They laid special stress on the statement that the cells did not make any movements toward the bacilli, nor was there any migration of bacilli toward the cells observed. No changes in the tissue elements induced by the bacilli could be described, and the presence of the bacilli within the cells did not stimulate the formation of giant cells.

MATERIAL AND METHODS

For cultivation with tubercle bacilli I chose the lymphoid tissue of the mesenteric lymph nodes, the omentum and the common loose

[19] Jour. Exper. Med., 1920, 31, p. 293.
[20] Ibid., 1916, 23, p. 265, 283.
[21] Am. Rev. of Tuberc., 1922, 6, p. 21.

intermuscular connective tissue of adult rabbits. Two strains of tubercle bacilli of the human type were used. The one recently isolated from sputum, passed through a guinea-pig and proved to be highly virulent; the other, originally also derived from sputum, grown for many generations on glycerol agar. It had a low virulence for guinea-pigs, but grew rapidly on artificial mediums.

The explantation technic was the usual technic described in my previous papers.[22] The tissue was always kept on round coverslips and as nutritive medium clotted bloodplasma from the carotid of young rabbits, with the addition of an equal amount of bone marrow extract, prepared with Ringer solution, was used. In some cases lithium carmin was added to this medium.

Usually, in order to give the tissue a growing start, it was first cultivated for about 3-5 days without bacilli; then the culture was opened, the round slip with the tissue washed in Ringer's solution, covered with a new drop of nutritive medium and inoculated with tubercle bacilli. The method of procedure included taking a loopful of bacilli from the agar surface and triturating it in 3 c c. of Ringer's solution; then a trace of this emulsion was transferred into the culture by slightly touching the edge of the spreading tissue with the end of a thick, straight platinum wire. In many cases, however, the tissue was inoculated with tubercle bacilli at the time of the first explantation.

It is self-evident that in each set of cultures only a part of them was inoculated with bacilli, while the rest was left for the purpose of control to develop without bacilli. Every fourth or fifth day the cultures with the tubercle bacilli were transplanted in the usual way, without disturbing the tissue, simply by taking off and washing the round coverslip. The longest time culture and bacilli were allowed to interact was three weeks. The phenomena most important from the morphologic point of view were usually found during the second week of growth outside the body.

The observation in living condition gives interesting results. But as the fibrin usually is not liquefied and the tissue layer is always relatively thick, the use of an adequate histologic technic is absolutely indispensable for a critical study of the behavior of the tissue elements toward the bacilli. Here again I applied my technic described in previous papers.[22] For fixation, Zenker formol was used, and in some

[22] Arch. russes d'Anat. d'Histol. et. d'Embryol., 1916, 1; Arch. f. mikrosp. Anat., 1922, 96, p. 494; Contributions to Embryology, Carnegie Inst., in press; Ztschr. f. wiss. Mikrosp., 1909, 26, p. 177.

cases, Champy's osmic solution. The serial celloidin sections, fastened on slides, were stained with Ziehl-Neelsen's carbol-fuchsin; the stain, covering the slide, was heated over a small flame until vapors arose, and left at room temperature for about 30 minutes. For differentiation, Orth's discharging fluid (1% HCL solution in 70% alcohol) was used for about half a minute. After that the slides were placed in distilled water for 10-24 hours for washing out the acid. Then the nuclei of the tissue were stained with a very weak solution of Delafield's hematoxylin during 24 hours; distilled water for 10-20 hours followed next, and finally the tissue was counterstained with the usual eosin-azure solution.

GENERAL DATA

The nutritive medium of my cultures seems to be favorable for the tubercle bacilli.

In all cases in which the nonvirulent strain mentioned above was used, they formed spherical colonies of various size 5 or 6 days after inoculation, the largest of them easily seen with the naked eye as gray dots scattered around the inoculated place and mixed with proliferating and spreading tissue elements. Coinciding with the description of Smyth, the bacilli in these colonies, especially at their edges, were most typically arranged in loosely twisted skeins of threads.

In the cultures of lymphoid tissue where, as I have shown, considerable migration of the various cells takes place and not only the peripheral, new tissue, but the explant itself may become very loose,[22] the tubercle bacilli are found scattered everywhere throughout the spreading tissue. On the contrary, in the case of the omentum and especially the intermuscular tissue, the bacterial colonies remain mostly on the surface of the explant, forming here also large colonies, and usually do not penetrate into the interior of the tissue or do it very slowly, except in places where they have been mechanically thrust into it by the platinum wire at the time of inoculation.

In the later stages the colonies sometimes reach an enormous size and cover large, irregularly outlined areas, surrounded by disseminated smaller colonies of spherical shape. However, even in these extreme cases, everywhere between and in these dense colonies of bacilli great quantities of living and motile cells could be discerned. In the cases in which the tissue explant was inoculated at once, these extreme conditions naturally were reached sooner than when the inoculation

was made at the first transplantation of the culture, and therefore the cases of the first type were less favorable for the study of the histogenesis of the tuberculous process in vitro.

The tubercle bacilli of the other highly virulent strain mentioned previously, behaved somewhat differently. They also multiplied in the tissue cultures, as can be seen in the fixed and stained preparations described below, but the rate of their proliferation was far lower; they never become numerous, do not form compact colonies, and therefore are not easily distinguishable in the living cultures. The cells of the rabbit tissues overpower and destroy them rapidly, so that 7 or 8 days after inoculation usually only traces of them could be discovered in the cultures on stained preparations. However, the quality of the cell reactions was identical in all cases.

The best pictures of the relations between the cells and the bacilli in the cultures, especially in the case of lymphoid tissue, are usually found at the edge of the explant, in the zone of newly formed tissue, with its loosely arranged cells penetrating into the fibrin. Another favorable place is the layer of liquid, which covers the explant and always contains great numbers of freely floating cells as well as bacterial colonies. When the bacilli were introduced into the explanted tissue itself, the typical tuberculous process develops here in exactly the same way as in the organism. Such conditions are described below for the omentum.

On the average, the ultimate fate of my cultures in the cases of the nonvirulent bacilli was a complete overgrowth of the tissue by the bacilli after three weeks of life in vitro. The excess of the bacilli could not be removed, and further observation of the cultures therefore did not present anything of particular interest, although living cells always could be found up to the last moment among the bacilli. In cases in which the highly virulent strain was used, the tissue, on the contrary, finally got rid of the bacilli, and its further development also was of no special interest.

The simultaneous symbiotic development of tubercle bacilli and tissue cells in vitro offers a striking difference in comparison to the behavior of the same cells toward other micro-organisms. It is well known that in cases in which tissue cultures accidentally become contaminated with saprophytic micro-organisms the cells prove absolutely unable to resist the obnoxious influence of the intruders. They may display a certain amount of phagocytosis toward the micro-organisms,

but they always degenerate rapidly. In cases in which different species of pathogenic micro-organisms were used (Smyth,[20] Lewis [19]), the cells and the bacteria also usually could not be kept alive together for any appreciable amount of time. In the presence of tubercle bacilli, on the contrary, the tissue cells, as we see, not only do not perish, but they display a series of reactive changes, partly of undoubtedly progressive character, which represent an exact duplication of the specific tuberculous inflammatory process, as it is found in the organism. This is the reason why I have chosen for the title of the present publication the expression "tuberculosis in vitro."

LYMPHOID TISSUE

The changes undergone by the lymphoid tissue while cultivated in vitro under usual conditions and in blood plasma with tissue extract, I have described in a series of articles profusely illustrated.[22,24]. I have shown that in these cultures the transformations of the various cell types can be followed with great accuracy. The cell types are: (1) Fibroblasts. They proliferate, grow radially as spindle shaped cells into the plasma and form the framework of the new tissue zone, surrounding the explant. (2) Endothelium of the blood vessels. It partly produces new capillary sprouts, partly becomes transformed into fibroblasts. (3) Reticular cells. They are mobilized as large, proliferating, ameboid and phagocytic, sometimes epithelioid polyblasts or macrophages, storing dyes, forming, in the presence of foreign bodies, through fusion, multinucleated giant cells, and sometimes accumulating in large numbers in the liquid on the surface of the explant. They are easily distinguishable by their pale, irregular nucleus, their ameboid protoplasm and the typical pigment granules, staining green with eosin-azure. (4) Lymphocytes. A part of them migrate into the surrounding medium and then degenerate; a part remain in the explant and in the loose new tissue zone and here continue to proliferate and transform themselves into plasma cells, into ameboid phagocytic polyblasts or into granulocytes.[23]

The same types of elements are encountered in the cultures, inoculated with tubercle bacilli, but their transformations have here specific peculiarities. Granulocytes are of common occurrence in the earlier stages, beginning with the fourth day. Later, however, as the tuberculosis picture develops, they mostly degenerate, apparently being

[23] Maximow, A.: Arch. f. Mikrosp. Anat., 1923, 97, p. 283.
[24] Arch. f. Mikrosp. Anat., 1922, 96, p. 494; 1923, 97, p. 283.

sensitive to the presence of the bacilli. Their remnants, staining deeply with eosin, can be found easily among the other elements, especially in the foci of caseous degeneration (fig. 22, *d*).

In the same way as in cultures without bacilli the lymph sinuses often are found enlarged and show a distinct framework of fibers with recticular cells in their lumen. If tubercle bacilli find their way into such cavities, instructive pictures of the behavior of the fixed reticular cells toward the bacilli can be seen (figs. 1 and 2).

Reticular Cells.—The chief rôle in the cultures of lymphoid tissue with tubercle bacilli is undoubtedly played by the reticular cells. They are mobilized as usual and part migrate into the surrounding medium, part stay in the explant or in the zone of new tissue. The ameboid cells in the plasma are usually the first to meet the bacilli. But in the case of lymphoid tissue, the bacilli, as I have mentioned, soon penetrate everywhere into the interior of the explant and therefore the reticular cells, remaining here, also in many places come in contact with the bacilli, sometimes even keeping their fixed position in connection with the fibers of the reticulum unchanged (figs. 1 and 2, *rc*).

Wherever the reticular cells meet the bacilli, phagocytosis promptly occurs. Another specific result of the infection is mitosis (fig. 1, *rc'*) and a peculiar hypertrophy of the cells, often attaining an enormous degree and concerning especially the nucleus and the cytocentrum; the latter phenomenon distinguishes this hypertrophy from what is found in noninoculated cultures. The huge nucleus acquires a regular oval shape with heavy smooth outlines instead of the former pale, folded membrane; the scarce small chromatin granules increase in quantity and size, and a large central nucleolus appears (figs. 5, 6, 7). The cytocentrum comprises a group of multiple centrioles, distinct even after the eosin-azure stain, and a large, pale, slightly acidophilic, homogeneous or faintly granulated attraction sphere, occupying the middle of the cell body and pushing the nucleus to the periphery (figs. 5, 7); the sphere is always free from inclusions or engulfed bacilli, which are scattered around the sphere in the peripheral, basophilic layer of the cell body, together with pigment granules and small fat droplets. Such elements always store large quantities of carmin, if it is present in the medium.

The described changes of the reticular cells are undoubtedly due to a specific chemical stimulation originating in the tubercle bacilli. Usually all the reticular cells in the whole culture, even those not coming in direct contact with the bacilli, show them, although in the cells con-

taining bacilli they are particularly conspicuous. This chemical influence operates at a distance and cannot possibly depend on substances of the body of the bacilli themselves, as the effect is seen already in young cultures shortly after inoculation when the number of the bacilli present is yet small and when no traces of their degeneration can be seen.

Reticular cells containing a various quantity of bacilli in their protoplasm are the most characteristic feature of the cultures now under consideration in the early stages after inoculation as well (4-5 days) as in the later, when usually the largest specimens can be found. They look very different in every special case. If they keep their fixed position on the reticulum and their irregular angular or stellate shape, as happens for instance in the enlarged sinuses mentioned above (figs. 1 and 2, *rc*), they may at once become transformed into typical epithelioid cells. The pictures correspond exactly with the first stages of experimental tuberculosis of the lymph nodes, as described by Joest and Emshoff.[16] Sometimes large, pale reticular cells containing bacilli are seen in the midst of dense crowds of perfectly normal proliferating lymphocytes, as it is reproduced for instance in fig. 7 of the article of the authors just named.

In most cases, however, the reticular cells are first mobilized to free ameboid polyblasts and then enlarge and acquire the epithelioid character. In the explant and in the denser parts of the zone of new tissue such large epithelioid cells usually remain spherical, or, if they assemble in large crowds (see below), they assume a polyhedral form through mutual pressure (fig. 21). In the looser areas of the growing and spreading tissue, in the fibrin, and in the layer of liquid on the surface of the explant, the large, motile reticular cells are either also regularly spherical (fig. 7), or they show a surprising variety of ameboid forms, the pseudopodia being very long, slender and branched (fig. 3), or massive and rounded at the edges (fig. 4), or digitated (figs. 5 and 6), etc. Sometimes the bacilli may be seen located in the slender, branched pseudopodia. Usually, however, they are confined, together with the other inclusions, to the layer of basophilic protoplasm, surrounding the large sphere, whereas the pseudopodia consist of a homogeneous, pale, highly motile ectoplasm and are free from bacilli (figs. 5 and 6).

As I have mentioned above, Smith, Willis and Lewis are strictly opposed to the idea of a real active phagocytosis of the tubercle bacilli by the cells of the embryo chick in vitro. The bacilli behaved in their experiments as any other foreign bodies of similar size and consistency

would do; they came in contact with the cells in a purely accidental way, and their entrance into the protoplasm was dependent merely on the consistency of the latter, the composition of the foreign body and the position of the bacilli in relation to the surface of the cell, i. e., on physical factors.

It cannot be doubted that the surface energy plays an important rôle in every case of phagocytosis. If cells of a certain type engulf certain foreign bodies in larger quantities than do other cells under the same external conditions, this must evidently be due chiefly to the especially favorable physical properties of the surface of the respective protoplasm, perhaps partly also to the greater motility of the cells, which brings them in contact with larger quantities of bodies in the same time unit than is the case with the other cells. The reticular cells in our cultures undoubtedly represent a case of this kind. The idea of true active phagocytosis includes the assumption that the cell does not come in contact with the foreign body in a merely accidental way, but that there is a kind of attraction of the cell by the foreign body, whatever the nature of the attractive force may be. In inflammation in the organism the leukocytes and lymphocytes are attracted by foreign bodies or by the soluble substances produced either in the damaged tissue itself, or in and by the bacteria. The same is the case, I believe, in our case of tissue cultures inoculated with tubercle bacilli. The colonies of the bacilli, especially the larger ones, are usually surrounded by dense crowds of mobilized, polyblastic reticular cells of various size, and the movements of the ameboid elements toward the bacilli can be easily watched in the living culture. Smyth has made the same observation.

Besides, the tubercle bacilli in tissue cultures have another remarkable influence on the free reticular cells, as already mentioned by Smyth. Throughout the whole colony, especially in the liquid covering the explant, even at a great distance from the bacilli the hypertrophying reticular cells, as well as the lymphocytes described below, display a marked tendency to cluster together and to form large groups freely floating in the liquid, or lying on the surface of the tissue, or located in the fibrin (figs. 7, 18, 19).

The varying number of tubercle bacilli in the reticular cells depends partly on the quantity of bacilli engulfed by the respective cell and partly on the changes undergone by the bacilli after phagocytosis.

On the one hand, one can often see, as Smyth [20] and Smith, Willis and Lewis [21] have already mentioned, unmistakable signs of intracellular

digestion of the bacilli in the reticular cells. They are sometimes surrounded by very large vacuoles, are broken up in smaller rods, assume the appearance of chains of cocci and are finally completely dissolved (fig. 19). Often, before being completely dissolved, the substance of the bacilli undergoes a typical degenerative change—it becomes transformed into a kind of pigment, showing after the fuchsin-hematoxylin-eosin-azure stain a bright golden yellow color, quite different from the green of the normal pigment (figs. 6 and 22); sometimes the pigment granules keep, for a while, the rod shaped form of the bacilli. All transitions can be seen in such cases from bright red, long, slender bacilli to brilliant yellow, irregularly granular inclusions. It is interesting to note, that sometimes during this process of intracellular digestion a third type of pigment inclusions appears around the cytocentrum of the ameboid epithelial reticular cells, where fat droplets and the bacilli are located—granules or drops of regular spherical form, showing a distinct yellow, but giving at the same time the osmic reaction—evidently a kind of lipochrome (figs. 8 and 9).

On the other hand, in other cells in the same culture the engulfed bacilli proliferate in the body of the phagocyte. In the progress of this intracellular proliferation first several sharply outlined spherical balls of densely crowded, coiled and twisted threads of bacilli are seen within the protoplasm. Gradually they fuse, and then the whole cell body appears crowded full with innumerable bacilli more or less radially arranged around the cytocentrum, which always remains free, as well as a very thin peripheral layer of protoplasm containing the nucleus or nuclei (fig. 21). It is remarkable that here, as in a typical case of symbiosis, the cell itself may not show any signs of degeneration for a long time. On the contrary, such cells can migrate and carry the micro-organisms all over the culture (fig. 4) and undergo quite an extraordinary hypertrophy, either remaining mononucleated (figs. 4, 21, *ep*) or gradually transforming themselves into giant cells (fig. 21, *gc*).

The reticular cells containing bacilli in all stages of their development, in the fixed and mobilized condition as well, and even while overcrowded with bacilli, often show quite normal mitotic division (fig. 8). The tubercle bacilli in the cell body, as far as it can be made out by approximate count, pass into both daughter cells in exactly equal numbers. This pertains in general to all dividing cells containing tubercle bacilli (fig. 13, *fble'*) and evidently must depend on the same factors

which cause certain rod shaped organoids of the cell, such as chondriosomes or parts of the reticular apparatus, to be equally distributed between the daughter cells.

In rare cases the larger epithelioid macrophages of reticular origin, containing bacilli, may show striking pictures of amitosis or irregular fragmentation of the nucleus (fig. 10).

The prominent rôle played by the reticular cells in the tuberculosis of the lymph nodes is fully established, and has been especially emphasized by Joest and Emshoff.[16] My experiments show that they become transformed in vitro in exactly the same way, as in the body, into typical epithelioid cells. Although the latter, with their large peripheral nuclei and hypertrophied cytocentrum, are so specific for the tuberculous process, they are to be looked on merely as a special type of polyblasts in general, the mononuclear nephrophagocytic elements playing such an important rôle in all the various types of inflammation. An especially close histologic resemblance and relationship exists between the epithelioid cells of the tuberculous lesions and the so-called "pus phagocytes," large polyblasts, described by Maximow[11] in the later stages of suppuration.

Fibroblasts.—Generally speaking, the fibroblasts behave exactly as in the common cultures of lymphoid tissue without bacteria (Maximow[24]). They are long, spindle shaped cells, which invade the plasma radially, proliferate, and form the loose network of the new tissue zone. Between them the tubercle bacilli are scattered, sometimes singly or in small clumps, sometimes in enormous dense masses. It is surprising how little the presence of the micro-organisms affects the fibroblasts. They usually look perfectly normal even when they are arranged in thin strands in the midst of a continuous network of bacilli skeins.

Occasionally bacilli can be found in their protoplasm; but this phenomenon is quite different from the phagocytosis by the reticular cells described above. Even in the cells surrounded on all sides by masses of bacilli always only single individuals or small loose groups of bacilli are found. Signs of intracellular digestion are extremely rare; furthermore, there are also no indications of an intracellular proliferation. Watching the living cultures, one can never notice any movement of the fibroblasts directed by the location of bacilli. Thus, in the case of the fibroblasts, we really have to assume a merely passive, accidental entrance of the bacilli into the cell body in the sense of Smith, Willis and Lewis.[21] Whereas the reticular cells are attracted by the bacilli,

and whereas the physical properties of the surface of their protoplasm are extremely favorable for the engulfing of the same, so that while we can speak of an active phagocytosis of the tubercle bacilli by the reticular cells, the behavior of the fibroblasts is completely passive and the bacilli enter the protoplasm only in rare cases with great difficulty.

The fibroblasts containing bacilli are also often found in mitotic division, with the same equal distribution of the bacilli between the daughter cells (fig. 13, *fblc'*).

I could not find in my cultures the transformation of fibroblasts in true epithelioid cells containing tubercle bacilli, as recently claimed by Castrén [2] for the tuberculosis in the human body. Often another phenomenon could be observed bringing about pictures which at a superficial examination might suggest such a transformation.

As described in my previous papers,[24] in the lymphoid tissue in the organism and in vitro as well new fibroblasts may constantly arise through differentiation from the embryonic reticular syncytium connected with the reticulin fibrous framework. The same syncytium gives rise also to new reticular cells. Under the influence of the stimulation, exerted by the tubercle bacilli, the fibroblast production sometimes may be found noticeably increased in the cultures, and coincidentally the cells show a rather unusual character—they remain in an embryonic condition. This does not mean that they become transformed into wandering cells; ameboid pseudopodia are never seen. The intensely proliferating cells have large or small, plump, angular basophilic bodies with sometimes very short, tapering, sharply pointed processes and with a distinct, acidophilic attraction sphere (fig. 12, *fblem*). In rare cases the cell body may transiently acquire even a spherical form, the typical fibroblast nucleus with the fine, pale chromatin granules and large nucleoli still always making the identification easy. In such a condition the fibroblasts closely remind one of embryonic mesenchymal cells or of the cells of a sarcoma. These fibroblasts of embryonic type often originate directly from the endothelium of blood vessels. The embryonic changes of the fibroblasts are typical for the cultures inoculated with tubercle bacilli. In common cultures this phenomenon could not be found.

It may be briefly mentioned that in some cases in which small particles of fat tissue surrounding the capsule of the lymph node were present in the inoculated culture, the fat cells showed a remarkable degree of activity, reminding one of their changes in aseptic inflam-

mation, as described by me in 1903.[11] The large fat drops in their body were rapidly dissolved, and they transformed themselves into fibroblasts of embryonic type.

Endothelium of Blood Vessels.—The transformation of the blood vessels which happen to be in the explant of lymphoid tissue is generally like that which I found in cultures without bacteria (Maximow [24]). A large part of the capillaries remains in the tissue as sharply outlined endothelial tubes. They may grow in various directions, forming new sprouts, radiating into the zone of new tissue and sometimes containing living or degenerating lymphocytes or polyblasts, detritus, and in many cases even small colonies of proliferating tubercle bacilli. Sometimes their lumen appears to be greatly enlarged, through absorption of liquid, and they become transformed into large, irregular cavities lined with thin squamous endothelial cells.

In other vessels, capillaries or even arteries and veins, the endothelial cells become transformed into proliferating fibroblasts, as I have already described for the cultures of lymphoid tissue without bacteria.[22,24] The same results are observed, as I found many years ago,[11] in common aseptic or purulent inflammation in the organism; the proliferating endothelial cells, especially in the small capillary veins, become detached from each other and recede from the vessel wall outward into the surrounding tissue, where soon they cannot be distinguished from common fibroblasts. Contrary to the dominant opinion, I never could find in my studies on inflammation any proof of the assumed transformation of ordinary endothelial cells of the blood, or lymph vessels into wandering phagocytic cells, i. e., into polyblasts.

In the tuberculous cultures the fibroblastic transformation of the endothelium of blood vessels is of widespread occurrence, in the early as well as in the later stages. In the central parts of the explant, and in the peripheral zone of new tissue, everywhere long strands of parallel, spindle shaped cells without any distinct lumen may be seen, running in all directions, sometimes surrounded by necrotic masses or by large colonies of bacilli (fig. 13, *fblc*). They can be quite easily traced back to unmistakable blood vessels, usually capillaries; sometimes they are seen growing out of the opening of a severed artery, with the circular muscular cells of the media still intact. The elongated, oval, pale nuclei of these cells first show a scarce quantity of chromatin and no distinct nucleoli. Gradually the latter appear, increase in size, and then the cell is absolutely similar to a fibroblast and behaves exactly in the same way (figs. 12 and 13, *fblc*). The parallel strands become dissociated

and the former endothelial cells mix with the common fibroblasts. The endothelial fibroblasts never produce ameboid phagocytic cells of the polyblast type and never become transformed into epithelioid cells. But they may contain tubercle bacilli, exactly as the fibroblasts of other origin, and coincidentally sometimes show mitotic division.

As I have mentioned, the fibroblasts in the tuberculous cultures often assume an embryonic character. This is especially true for the fibroblasts arising from the blood vessel endothelium. A typical place showing a large crowd of proliferating embryonic fibroblasts of endothelial origin is represented in fig. 12. The angular cells (*fblem*) with the acidophilic sphere often are arranged in the fashion of a loose epithelial pavement. In rare cases they may even round off more or less completely and occasionally also contain bacilli (fig. 12, *x*). Nevertheless, they are always easily distinguishable from the true epithelioid cells derived from the reticular cells or from the lymphocytes. In the first case, the cell body usually contains the typical greenish pigment (fig. 12, *cp*); in the second, the nucleus is an unmistakable sign of its origin.

Lymphocytes.—In 1902 I showed that the emigrated lymphocytes on the field of inflammation transformed themselves into ameboid phagocytic cells, the polyblasts. I gave them this name because they displayed an amazing variety of prospective potencies and produced many other cell types found in the inflamed area. Recently [24] I succeeded in obtaining in cultures of lymphoid tissue the same transformation of the lymphocytes of various types, small, medium and large, into polyblasts and various other related cell types.

In the cultures of lymphoid tissue, inoculated with tubercle bacilli, the lymphocytes which have migrated into the surrounding fibrin as isolated elements ordinarily perish, exactly as in common cultures. The lymphocytes in the explant, in the zone of new tissue and in the liquid, covering the culture, on the contrary, show progressive development and proliferation. The presence of tubercle bacilli seems to exert a stimulating effect on them; it is difficult in this respect to make exact statements, but one has the impression that their rate of mitotic division is increased in comparison with the ordinary conditions of life in vitro. They always show intense ameboid movements.

The lymphocytes are found in the cultures either in dense masses, if parts of follicles have been explanted, or in irregular smaller and larger groups scattered everywhere, together with the tubercle bacilli. Wherever they are freely floating in a liquid medium, they show a

marked tendency to assemble in clusters (fig. 7, 15, 16), especially around large free macrophages of reticular origin or around giant cells (fig. 7 and 20). The clusters are often found on the surface of the coverslip; but their place of predilection is the layer of liquid covering the explant.

The tendency to cluster together with large cells of polyblastic type is found also in lymphoid tissue cultures without bacilli,[24] and it is found even in normal embryonic development of blood forming organs, for instance in the vessels of the yolk sac.[18] But in the presence of tubercle bacilli this tendency manifests itself with surprising regularity—a fact, already mentioned by Smyth.

The myeloid, granulocytic elements arising from the lymphocytes, as I have mentioned, soon degenerate and cannot be found alive after the 8th or 9th day.

The development of plasma cells is also inhibited, and one practically never sees the lymphocytes differentiate in this direction. It is well known that ordinary plasma cells are not found in the vicinity of fresh tubercles in the organism.

In the later stages, 10-12 days of life in vitro, 4-6-8 days after inoculation, a surprising quantity of typical, large, proliferating, basophilic lymphocytes is usually found (fig. 16, 11*m*), especially in the growing zone of new tissue. They develop directly through hypertrophy from small lymphocytes, and it is easy to find all the transitional forms.

The transformation of the various types of lymphocytes into large ameboid phagocytic elements of polyblast character can also be easily observed (fig. 14, *plb*). The pictures are identical with those I found in cultures without bacilli, and they naturally correspond to the same process in common inflammation, as observed in the organism.[11, 18]

As soon as the polyblasts of lymphocytic origin acquire a sufficient size they cannot be positively distinguished from polyblasts originating from the fixed, reticular cells, except when the latter still keep their typical greenish pigment. It is clear, therefore, that after having undergone a sufficient hypertrophy, the lymphocytes can become transformed into epithelioid cells, which, as I have already emphasized, are only a special type of polyblasts and which accordingly can arise from two sources—from the fixed, reticular, or resting wandering cells, or from lymphocytes. As this transformation of lymphocytes into phagocytic polyblasts is an established fact confirmed by many investigators—it can

be occasionally followed in the living culture—it is by no means surprising, that on fixed and stained preparations of the inoculated cultures often transitional forms, still showing characters typical for the lymphocyte, may be found containing engulfed tubercle bacilli (fig. 14 and 18, *plb*).

But it is interesting that even unchanged, perfectly typical, highly basophilic, large or medium sized lymphocytes, especially when assembled in clusters and intensely proliferating, if they happen to come in contact with tubercle bacilli, also occasionally engulf them (fig. 13, 15, 16 *lb*). Phagocytosis of tubercle bacilli by lymphocytes has never been seen in tuberculous lesions in the organism. This process, as in the case of the fibroblasts, is probably no active phagocytosis; I never saw lymphocytes move toward the bacilli in the living culture; this is a passive, physical phenomenon. The engulfed bacilli are sometimes surrounded by clear vacuoles (fig. 16, *lb*), but they do not seem to undergo any changes as long as the lymphocytes keep their character unchanged. But as the lymphocytes become transformed into polyblasts and epithelioid cells, and, as we shall see below, even play a rôle in the formation of giant cells, evidently the bacilli contained in their protoplasm may finally, at least theoretically, be digested and destroyed in the same way as described above for the epithelioid cells of reticular origin.

Formation of Tubercles and Giant Cells.—The formation of small, more or less sharply outlined agglomerations of epithelioid cells, the tubercles, especially if they contain giant cells of the Langhans type, with peripherally assembled nuclei, has to be looked on as the most characteristic anatomic symptom of the tuberculous lesions. Although sometimes they may be absent in the presence of tubercle bacilli and be substituted by a more diffuse granulation, yet without these two elements one cannot properly speak of a typical tuberculous process.

Observing the inoculated cultures of lymphoid tissue in living condition beginning, on the average, with the fourth day after inoculation, when the scattered colonies of the bacilli become easily distinguishable, one finds that smaller or larger cell foci develop in many places in the explant itself and in the zone of new tissue, especially on the surface of the coverslip, in the fibrin and in the layer of liquid covering the culture (fig. 18 and 21). The majority of the cells, after fixation and staining, prove to belong to the reticular cells described above; but between them lymphocytes are also always found in large though varying quantities, and show hypertrophy and transformation into polyblasts

(fig. 18, *plb*). Fibroblasts are also often present, but they remain passive, do not round off or assemble together, and remain at the periphery of the foci.

The formation of these cell foci is chiefly the result of a very slow movement of free cells directed toward a certain point. If, as often happens, the cells are assembled around a central colony of bacilli and engulf them, this process can be looked on as a phase of active phagocytosis. But the presence of a special central colony is not necessary. The bacilli are usually irregularly scattered between the cells and sometimes may be very scarce or totally absent at the exact place where the cells gather. As I have emphasized, all reticular cells and lymphocytes in an inoculated culture display a remarkable tendency to assemble in clusters, even if they do not come in direct contact with the bacilli. This tendency is shown even by the reticular cells still attached to the fibers of the reticulum.

The number of the cells in the clusters increases partly through mitotic division. Besides, the cells, as we have seen, hypertrophy, assume an epithelioid character, and sometimes, through mutual pressure, become polyhedral (fig. 21). In this way the cell foci may acquire considerable size. Most of the cells contain bacilli in various, sometimes enormous, quantities (fig. 21). Their structure and transformation have already been described above.

These agglomerations of reticular cells transformed into epithelioid cells, and of lymphocytes in large part gradually undergoing the same transformation, must be compared with true tubercles. The absence of a circulation in the tissue cultures does not affect the microscopic picture of the tuberculous process, evidently because the miliary tubercles in the organism, as is well known, also do not contain any blood vessels.

The formation of giant cells of the Langhans type is of constant occurrence; they are never missing in the inoculated lymphoid tissue cultures. This has already been correctly observed by Smyth.

In common cultures of lymphoid tissue without bacilli, multinucleated giant cells are frequently found, but here they always are formed either on the surface of the coverslip, or in contact with some foreign bodies, such as cotton fibers, etc.[24] They have been seen in cultures of various other tissues (Lambert,[25] Lambert and Hanes,[26] W. H. Lewis and L. T. Webster.[27] These are the common foreign body giant cells observed in various inflammatory processes in the organism.

[25] Jour. Exper. Med., 1912, 15, p. 510.
[26] Virchows Arch. f. path. Anat., 1913, 211, p. 89.
[27] Jour. Exper. Med., 1921, 33, p. 349.

The origin of these elements in the organism and in vitro is described in different ways, both as to the nature of the cells serving as material and as to the mode of formation. Using a special technic in 1902 I demonstrated that the foreign body giant cells, as a rule, arise in aseptic inflammation from ameboid polyblasts through fusion of separate mononuclear cells; during this process the cytocentra of the single cells always assemble in the middle of the new giant cell body and also fuse, producing a large attraction sphere with one or several groups of numerous centrioles, whereas the nuclei are arranged in the peripheral zone of the cell body.

The giant cells of the Langhans type are histologically almost similar, also showing a large homogeneous central area and peripheral nuclei. The assumption by some authors that this area is a degeneration of the central part of the cell body and a typical phenomenon of tuberculosis proved to be wrong. As Wakabayashi,[3] Wallgren [1] and Castrén [2] have shown, and as I shall presently describe, this homogeneous clear area of the Langhans giant cells is also an attraction sphere with a multitude of centrioles, as in the foreign body giant cells, but of an exceptionally large size and homogeneous aspect.

The formation of typical giant cells of the Langhans type in inoculated cultures of lymphoid tissue can be observed to best advantage during the early stages, for instance 4-6 days after the inoculation of the tissue with tubercle bacilli. In several cases I was able to follow this process in living condition step by step, together with the clustering of the cells and the formation of tubercle-like cell crowds. But in the majority of the cultures the tissue layer in the peripheral growing zone and the cell clusters are not transparent enough to allow an exact interpretation of the microscopic picture in vivo; the fixed and stained preparations are, as a rule, of an extraordinary clearness.

The chief source of the giant cells are the reticular cells, and in most cases they are formed after the latter have been mobilized as free elements and have become changed into epithelioid cells. But they also can arise from smaller, polyblastic, or even directly from fixed reticular cells still attached to the reticulum; in this case the free epithelioid stage is skipped.

Fig. 18 demonstrates an instructive case of the first stage of giant cell formation in the liquid layer covering the culture four days after inoculation of the tissue with the nonvirulent strain of tubercle bacilli; the tissue had a start of 5 days. The majority of the cells, forming a

large cluster, are typical young polyblasts, which could not as yet possibly be called epithelioid cells; they show a distinctly ameboid protoplasm, containing a few fat droplets and vacuoles and an irregularly shaped nucleus with few chromatin granules. As it often happens, the cells, having arisen from reticular cells through mitotic division, have lost the greenish pigment usually so characteristic. Many of the cells contain engulfed bacilli. In several places (fig. 18, *gc*) the limits between the crowded elements become indistinct, and the cell bodies fuse to large spherical masses of protoplasm. In this case the process was observed in the living condition before fixation.

As soon as the cells fuse, the cytocentra assemble in the middle of the new large body and here one or several groups of centrioles can be seen surrounded by a growing attraction sphere. The nuclei arrange themselves at the periphery in the form of a wreath and for a while keep their internal structure unchanged. The bacilli also recede to the periphery. It is clear that this process of giant cell formation resembles very much the formation of common foreign body giant cells, such as I described in 1902.

Another case of the same process is seen in fig. 19, taken from a culture immediately inoculated with the virulent strain of bacilli and kept in vitro for 6 days. Here the cells are typical large epithelioid cells; they do not show distinct pseudopodia, but contain a large quantity of fat droplets, engulfed remnants of dead cells and bacilli, surrounded by vacuoles and apparently in various stages of intracellular digestion. The gradual disappearance of the cell limits in the group is seen with striking clearness.

A case of another character is represented in fig. 2, from a culture inoculated without delay with the nonvirulent bacilli and kept in vitro for 5 days. The area illustrated is directly adjacent to that of fig. 1. Here we see the immediate result of the fusion of two reticular cells, still keeping their fixed position in connection with the reticulum and containing a few fat droplets, a large vacuole and a clump of engulfed bacilli. In the middle of the binucleated cell, between the two nuclei which had not had time to change their internal structure, is clearly seen the acidophilic sphere with the faintly stained centrioles. With continued development such a binucleated cell would become free and would assume a structure such as that in the cell in fig. 11; here the two nuclei are large and regularly oval and contain a conspicuous nucleolus whereas the center of the cell body is occupied by a large

acidophilic sphere surrounded by the basophilic, still slightly ameboid, protoplasm with bacilli and green pigment granules.

The giant cell formation may stop after 2 or 3 cells have fused together, and such large transitional forms, between epithelioid and giant cells, with a central sphere, two or three nuclei and bacilli and pigment granules in the peripheral protoplasm are common (fig. 11). Sometimes they are crowded with bacilli, exactly as the mononuclear epithelioid cells are (fig. 21, *gc*); usually this does not happen with typical large multinucleated giant cells of the Langhans type. The cells, containing 2 or 3 nuclei, probably can enlarge later through additional fusion of other mononuclear cells and thus finally still furnish multinucleated cells.

The usual final result of the process described are the typical giant cells of the Langhans type, scattered everywhere in the infected cultures 4-5 or more days after inoculation and sometimes remaining apparently unchanged and without signs of degeneration up to the latest stages observed. If they float freely in liquid, they are spherical; but as they usually, especially the larger ones, are either adjacent to the glass or are surrounded by other tissue elements, their form varies considerably, as in the tuberculous lesions in the organism. The later the stage of the culture, the larger they usually are. In fig. 20, one of the most common types is represented, exactly similar to the giant cells of a young miliary tubercle in the organism. The center of the cell body is occupied by two large spheres with centriole groups, which as yet have not had time to fuse together. The nuclei are fixed in the process of the final rearrangement so as to occupy a peripheral position, and they now show the typical structure of a giant cell nucleus—vesicular appearance with sharp contours and one large nucleolus in each. In the peripheral protoplasmic zone groups of bacilli and a few green pigment granules are scattered. The latter clearly indicate the origin of the giant cell from reticular cells.

The intracellular digestion of the engulfed bacilli in the epithelioid cells is continued and probably highly increased after their fusion to multinucleated giant cells. Therefore the number of the bacilli in the typical giant cells of the Langhans type is never very large, and no excessive intracellular proliferation occurs, as in the mononuclear cells previously described. Often giant cells are found with the same golden yellow pigment inclusions, resulting from destroyed bacilli, as in the mononuclear epithelioid cells.

The rôle of amitosis in the formation of the tuberculous giant cells is inconspicuous. As I have mentioned, constricted and fragmented nuclei are occasionally found in single epithelioid cells (fig. 10) and at times in already formed giant cells (fig. 17); but they are scarce in comparison with the pictures showing the various stages of fusion.

The giant cell in fig. 17, taken from an older culture of 14 days, 10 days after inoculation, is only partly seen, because it is cut in several sections. The huge irregular body contains a great quantity of nuclei, partly showing amitotic fragmentation. The protoplasm, containing vacuoles and small fat droplets, is full of tubercle bacilli. In such large giant cells, again exactly duplicating the conditions in the organism, the regular concentric arrangement of the nuclei with the other parts is effaced, and the cytocentrum is not clearly visible.

The interesting peculiarity of the cell in fig. 17 is due to the contamination of the respective culture with saprophytic cocci that are also phagocytized by the giant cell; but, whereas the tubercle bacilli do not seem to affect the vitality of protoplasm or nuclei, the cocci have caused in the area where they are accumulated (*y*), a rapid degeneration of the nuclei (*z*). The different reaction of the living substance of the cell to the toxic products of different bacteria here manifests itself within the confines of a single cell individual.

An unsolved problem is, as we have seen, the participation of the lymphocytes in the formation of tuberculous giant cells. As has happened in many other similar cases in science, the extreme standpoints must be replaced by an intermediate position.

From the description we have seen that the lymphocytes easily become transformed into polyblasts (fig. 14, *plb*) and later may acquire an epithelioid character. As I have already pointed out in another paper,[24] they usually can be distinguished by the absence of the pigment granules, staining green with eosin-azure. It is true that this criterion sometimes fails, because the mobilized reticular cells, through proliferation and diminution in size, may also lose their pigment. But the close genetic relationship between the lymphocytes and the reticular cells is a well established fact.

If, then, the lymphocytes can become transformed into epithelioid cells, evidently they must, secondarily, play an important rôle in the production of the giant cells. In most cases this takes place after their transformation into large, epithelioid polyblasts, and therefore in the clusters showing giant cell formation through fusion, as described above, one cannot discriminate between the polyblasts of different origin.

In some cases, however, as in fig. 18, in which giant cell formation evidently occurred with unusual speed, the cell clusters contain cells which cannot be looked on as other than typical lymphocytes, fixed in various stages of their transformation into polyblasts and epithelioid cells (fig. 18, *plb*). They are scattered between the polyblasts of reticular origin and rapidly join them, fusing together and largely contributing to the giant cell formation.

My conclusion, therefore, is that the Langhans giant cells in the lymphoid tissue are formed partly by epithelioid polyblasts of reticular origin, partly by lymphocytes; the latter either first hypertrophy and become indistinguishable from the former, or the process is abbreviated, the epithelioid stage is skipped, and the lymphocytes join the other elements as common young ameboid polyblasts (fig. 18).

I could never find any indications of an endothelial or fibroblastic origin of the giant cells. Even the proliferating fibroblasts of the embryonic type described above (fig. 12, *fblem*) do not seem to play any rôle in this process. The rôle of the endothelium in tuberculosis I shall discuss later.

The principal mechanism of the giant cell formation is the fusion of mononuclear cells. This phenomenon is to be looked on as a further step in the process of clustering of the cells and of tubercle formation under the influence of the chemical stimulus originating in the bacilli. It must be due to a sudden decrease of surface tension in the respective cell bodies.

Immediately after fusion a reorganization of the new cellular unit on a larger scale takes place. The highly differentiated cytocentrum, the result of the gradual fusion of the centra of the individual cells, occupies the middle region of the body, whereas the nuclei, together with the inclusions and the bacilli, assemble at the periphery.

There is no fundamental difference between the formation of the tuberculous and the common foreign body giant cells. The former are to be looked on merely as a special type of the latter, acquiring their peculiar structural character under the influence of some diffusible chemical substance produced by the bacilli. They may therefore arise without a direct mechanical contact with the bacilli and may not contain them at all. This is especially manifested in the case of the loose connective tissue, as described below.

If the inoculated cultures happen to contain foreign bodies, as for instance cotton fibers, transitional forms between the two types of giant

cells are found in direct contact with these bodies. They are large, irregular, protoplasmic masses, ensheathing the body and containing numerous nuclei scattered without any visible order—tubercle bacilli, green pigment granules, fat droplets and vacuoles, but usually no distinct cytocentra. In connection with this it may be recalled, that by injecting into the tissues, especially in the serous cavities, a suspension of small inert foreign bodies, like infusorial earth or lycopodium spores, so-called pseudotubercles can be produced, also containing epithelioid polyblasts, lymphocytes and giant cells, but without tubercle bacilli and without caseation.

Caseous Degeneration.—The usual fate of the elements constituting a tubercle is a peculiar regressive change, the so-called caseous degeneration.

In tissue cultures, local degeneration and necrosis of single cells or of whole areas of tissue are of widespread occurrence. Therefore, it is not easy to determine whether a necrotic focus found in an inoculated culture is a case of caseation or merely of common coagulation necrosis, the more so as in the organism the microscopic picture may also be almost similar in both cases.

However, in cultures with advanced formation of tubercles and giant cells, beginning with the 7th day after inoculation, foci of degeneration can be found showing a striking resemblance to typical caseous degeneration, as found in the organism (fig. 22). In the central region of a tubercle-like accumulation of large epithelioid or giant cells the nuclei, usually receding to the periphery of the cell bodies, assume an irregular form, shrink, stain very dark or very pale and finally are broken up into small fragments of chromatin, which are soon completely dissolved; it is a typical picture of karyorrhexis and karyolysis (*k*). The protoplasm in the meanwhile acquires a peculiarly pale, homogeneous aspect and contains, besides a various quantity of bacilli and yellow pigment inclusions, lumps of an acidophilic substance and, as is seen after osmic fixation, numerous fine fat droplets of more or less equal size. Often between these degenerating elements dead lymphocytes or dead granulocytes (fig. 22, *d*) are scattered.

The final result of these changes is a smaller or larger area of detritus, surrounded by living tissue, continuing its development and containing epithelioid and giant cells and colonies of bacilli. Often the detritus is invaded by strands of fibroblasts or fibroblast-like endothelial cells.

OMENTUM

The tissue of the omentum is complicated and contains many cellular types whose interrelationships are not yet settled. An analysis of these elements may be found in a recent paper by one of my students, A. Wjereszinski;[28] besides, experiments with tissue cultures of the omentum are now under progress in my laboratory, and their results will be published in a separate paper. For the sake of clarity, I may briefly enumerate the principal cell types of the omentum, playing a rôle in the experimental tuberculosis of this tissue in vitro.

(1) Fibroblasts. In the omentum they are somewhat different from the fibroblasts in other types of connective tissue, and, as I pointed out in 1902 and on various later occasions,[30, 18] they show a more embryonic character. (2) The mesothelium, the squamous cells, covering the surface. The problem of their relations to the fibroblasts is not settled. (3) The resting wandering cells or clasmatocytes, corresponding in their biologic characters to the reticular cells of the lymphoid tissue and especially numerous, in resting and mobilized condition, in the so-called milky spots; they are the chief source of the large free phagocytic cells of the peritoneal cavity, the "exudate polyblasts" of Wjereszinski, and in the tissue of the omentum itself all the transitional forms between these two cell types may be found. (4) Lymphocytes. Some are of local origin and some, as Wjereszinski has shown, emigrate from the blood vessels.

The behavior of the tubercle bacilli in an omentum explant, inoculated either at once, at the time of explantation, or at the first transplantation into fresh medium, is exactly the same as in the cultures of lymphoid tissue described above. The highly virulent bacilli do not multiply rapidly and are soon found engulfed by cells forming tubercles. The nonvirulent bacilli proliferate excessively and soon, 5 or 6 days after inoculation, form on the surface of the explanted membrane large, sometimes enormous, colonies.

Being a thin membrane, the tissue of the omentum always thrives very well in vitro, and, in the presence of tubercle bacilli, not only does it not show any inclination toward degeneration, but its elements, on the contrary, manifest typical and specific reaction changes, which are here, as well as we have seen in the lymphoid tissue, exactly similar to the changes of the omentum in tuberculous peritonitis.

[28] Haematologica, 1924.

During the first three days of life in vitro the tissue of the omentum surrounds itself with ameboid cells, emigrating into the fibrin and coming chiefly from the milky spots; they are partly lymphocytes, but mostly exudate polyblasts with an excentric kidney shaped nucleus and a highly developed cytocentrum. The number of the emigrating elements is exceedingly variable and is always very large in the neighborhood of the milky spots. On the surface of the tissue, adjacent to the medium, spindle shaped fibroblasts and mesothelial cells are seen rapidly invading the fibrin.

Beginning usually with the fifth day after inoculation, the ameboid exudate polyblasts, with a various number of lymphocytes, assemble in clusters on the surface of the tissue, where the bacillary colonies develop. This phenomenon is exactly similar to that described above for the lymphoid tissue. The polyblasts sometimes, although rarely, show mitotic division. They phagocytize and digest the bacilli and behave exactly in the same way as the mobilized reticular cells. They may undergo considerable hypertrophy, especially of their attraction sphere, and acquire the type of epithelioid elements, containing bacilli, fat droplets and pigment granules. Finally, the cell limits in the clusters in many places become indistinct, and the cells fuse to form giant cells with a large central sphere and a various number of nuclei and bacilli surrounding it. The lymphocytes, although being naturally much less numerous than in the cultures of lymphoid tissue, also participate in the giant cell production.

Often the fibroblasts and the mesothelial cells on the surface are found immediately adjacent to colonies of bacilli and in general behave passively, although they may contain in their protoplasm a varying, sometimes considerable, number of bacilli. In the omentum, however, it is difficult to exclude the possibility of the formation of epithelioid and giant cells by the fibroblasts, because the fibroblasts here are of an unusual, embryonic type and because they, as well as the mesothelial cells, easily contract and round off. Regarding the endothelium of blood vessels, I can exclude participation in the formation of epithelioid and giant cells with positiveness.

The tissue of the omentum, while cultivated in vitro, does not lose its normal architecture and is not as deeply altered and loosened as is the case with the lymphoid tissue. It remains in the explant as a wrinkled, irregularly folded, sharply outlined membrane. Nevertheless, the proliferating bacilli find their way into the tissue, partly, perhaps, because they are directly thrust into it by the platinum wire during

inoculation. Wherever they are found between the collagenous fibers and the various cellular elements, they invariably cause the development of nodules consisting of mobilized resting wandering cells transformed into epithelioid polyblasts and containing a various quantity of bacilli, usually phagocytized by the cells and in a more or less advanced stage of degeneration and digestion (fig. 23). Mitoses are seldom found in the nodules, so that their formation must be chiefly the result of a slow movement of the mobilized elements toward a certain point. Such bacilli-containing cell nodules in the tissue of the omentum are typical tubercles, although no formation of giant cells in them could be found.

In the omentum, as well as in the other tissues studied, the general influence of the presence of tubercle bacilli in the culture on all of the cells, even on those not in direct touch with them, was distinctly manifested. All the ameboid polyblastic cells showed a distinct epithelioid hypertrophy, an enlargement of the attraction sphere and a marked tendency to cluster together.

LOOSE CONNECTIVE TISSUE

The changes of the common intermuscular loose connective tissue of the adult rabbit in vitro have been described by me in a previous paper.[22] Here, as well as in other types of connective tissue, the fibroblasts penetrate from the edge of the explant into the fibrin and gradually form a zone of new tissue; the resting wandering cells (clasmatocytes) scattered in the tissue singly or, usually, arranged in small groups, migrate into the fibrin as polyblasts. But the majority of them remain in the explant and show accumulation of peculiar granular inclusions in the protoplasm.

In the inoculated cultures the intercellular substance always appears highly edematous, the collagenous fibers are swollen and not sharply outlined (fig. 24 and 25, *c*) and between them a large quantity of an amorphous substance is seen, after fixation showing a granular appearance. At the edge of the explant migration [illegible] growth of cells into the surrounding medium is seen. The a[illegible] of the elements in the interior of the tissue—the fibroblast network and the typical small groups of resting wandering cells—remains unaltered (fig. 24 and 25).

The bacilli multiply exuberantly on the surface of the explant, but, as has been mentioned above, penetrate into the edematous tissue either slowly or not at all. If small air bubbles are introduced into the jelly-like intercellular substance at the time of explantation, the tubercle bacilli usually are found surrounding them in dense masses.

On the surface of the explant occasional clusters of epithelioid cells, originating from the resting wandering cells, may be found, with the same behavior toward the bacilli as in the cultures of other tissues. However, as the edematous intercellular substance seemingly offers a great obstacle to the movements of the wandering cells, they mostly remain inside the explant, and the clusters mentioned are scarce and may be absent altogether. Single ameboid polyblasts containing bacilli are always found in the fibrin. The fibroblasts, growing from the surface penetrate between the colonies of bacilli and behave in the same passive way as in the other tissues described, and sometimes occasionally engulf bacilli.

Although, in most cases, the bacilli are practically absent in the tissue itself, all the cellular elements throughout the explant, keeping their position and arrangement unaltered, show remarkable structural changes (fig. 24 and 25). It is a most persuasive example of a chemical action exerted by bacilli on tissue cells at considerable distances and through layers of colloidal material.

The fibroblasts at first hypertrophy, then, after 2 or 3 days, show mitotic figures; consequently, after 9 days, they are more numerous, but again smaller. Their nuclei are richer in chromatin than usual, and the cell body acquires a compact angular or stellate form; the slender branching processes of the basophilic protoplasm extend everywhere between the swollen collagenous bundles and form a diffuse, continuous framework throughout the tissue, with the groups of resting wandering cells in the nodes (fig. 24 and 25, *fblem*). Most typical is the change of the cytocentrum in the fibroblasts. The attraction sphere hypertrophies and represents a distinct, acidophilic body and in the later stages, 10 or 11 days, it sometimes shows degenerative changes—formation of a group of coarse, irregular droplets of a substance, staining with eosin-azure a deep purple. Occasionally amitosis of the fibroblast nucleus is found, the result being binucleated fibroblasts.

The fibroblasts just described are almost similar to the proliferating fibroblasts in the inoculated cultures of lymphoid tissue and also may be considered as elements, having acquired some of their former embryonic characters.

The most striking changes, however, are undergone by the resting wandering cells (fig. 24, *b,h*). They enlarge excessively, their protoplasm, instead of the coarse granules of the cultures without bacilli, is

full of vacuoles and appears alveolar. The nuclei show either mitotic division (*h*) or amitotic constrictions (*b*), leading to the formation of cells with two or more nuclei.

Thus, in the common connective tissue, the resting wandering cells show the greatest sensitiveness toward the bacterial toxins. They respond to this stimulation with the same exactitude as the reticular cells in the lymphoid tissue, or the exudate polyblasts in the serous membranes and become also transformed into epithelioid cells. But, due to the presence of the edematous, jelly-like intercellular substance surrounding them from all sides, they are unable, under the conditions prevailing in vitro, to move and to assemble together; when tuberculosis develops in the loose connective tissue in the organism, the same intercellular substance is probably liquefied by enzymes, which are absent in vitro. Therefore, in vitro, at least in the stages at my disposal, no clusters and no tubercles are formed. All the cells keep their original position. The same reason accounts for the lack of giant cells. Instead of the usual giant cell formation through fusion, here where the cells remain widely separated from each other, only amitosis comes into effect and, accordingly, the giant cells remain comparatively small and contain few nuclei and no bacilli.

It is possible that if the symbiotic development of the connective tissue with the tubercle bacilli could continue longer, the hypertrophied resting wandering cells at last still would form typical tubercles, as we have seen them form in the omentum.

DISCUSSION AND CONCLUSIONS

The description indicates that if mammalian tissues are cultivated in vitro together with tubercle bacilli under suitable conditions, no degeneration and death of the cells occur, as always happens in contamination of a tissue culture with saprophytes, but that a kind of symbiosis is established. Both partners may grow and proliferate together for a long time (3 weeks); sometimes even single cells, alive and even dividing, are found in the midst of enormous masses of bacilli.

In the presence of tubercle bacilli the tissue elements always show a series of definite, specific, reactive changes. The whole complex of the phenomena observed in vitro gives an exact duplication of the tuberculous process as it is found in the infected organism, up to caseous degeneration; sometimes even a hint of a "healing" invasion of fibroblasts into the necrotic foci may be observed.

In the lymphoid tissue the reticular cells are the most active elements. Exactly as in the infected lymph nodes in the organism, they are mobilized, hypertrophy, divide and become transformed into large polyblasts, epithelioid cells, actively phagocytizing the tubercle bacilli. One of the most conspicuous changes in these cells is a peculiar hypertrophy of the cytocentrum.

The epithelioid cells assemble in clusters, corresponding to tubercles, which, according to the terminology of Joest and Emshoff,[16] could be called "primary" tubercles. Many of the cells fuse together, apparently because of a decrease of surface tension in their protoplasm, and in this way giant cells of the Langhans type are produced; the increase in the number of nuclei in the latter is partly due to amitosis.

The lymphocytes, as in common inflammation, also play an active rôle. They hypertrophy, become transformed into polyblasts, later sometimes acquire an epithelioid character and join the reticular cells in the process of tubercle and giant cell formation.

Some few bacilli may enter the protoplasm of the lymphocytes in a passive way. Later, when the lymphocytes are changed into epithelioid cells, active phagocytosis sets in.

The tubercle bacilli, engulfed by the epithelioid cells, are either digested, leaving in the protoplasm a peculiar yellow pigment, or they multiply excessively in the body of the phagocytes, overcrowding the protoplasm. Such cells, full of bacilli, may keep their vitality for a long time, move about and even divide mitotically. In the giant cells, the digestive power of the protoplasm is probably far higher, and therefore the number of the intracellular bacilli usually does not increase excessively.

The typical end result of the tuberculous process in the organism, the caseation, also can be observed in the cultures. In the larger clusters or tubercles, the epithelioid cells degenerate, showing diffuse accumulation of small, evenly distributed fat droplets, karyorrhexis and karyolysis, and finally detritus with occasional bacilli remains.

Similar facts can be observed in inoculated cultures of the omentum and simple connective tissue. In the first, the resting wandering cells are mobilized to epithelioid polyblasts, engulf tubercle bacilli and form typical tubercles. In the second, although the bacilli usually do not penetrate into the tissue, the resting wandering cells hypertrophy, are transformed into epithelioid cells and even small, atypical giant cells are formed through amitosis of their nuclei.

The fibroblasts do not take any part in the formation of tubercles and giant cells. They proliferate and assume an embryonic character, but remain sharply separated from the other elements; an exception might perhaps be found in the case of the omentum.

An important problem is the question of the rôle played by the endothelium in the production of the typical tuberculous formations. That the epithelioid and giant cells are the direct product of endothelial cells, is, in fact, a current, or even dominating, opinion, quoted everywhere in the textbooks on pathology; some experimental data seem to support it (Foot [14]). But, if we examine the facts and the literature more closely, we come to the conclusion that this dominating opinion needs correction. The problem is closely connected with another question—what is to be called endothelium?

If we confine the use of this name only to the squamous cells lining the common blood vessels—capillaries, arteries, veins and heart—and the lymph vessels, it will be difficult, if not impossible, to find any real proof for their assumed participation in the production of the elements of the tubercle. In the tissue cultures—as well as in suitably treated material of any inflammatory process—the transformations undergone by this true endothelium are quite easy to follow. The endothelial cells either keep their former arrangement in tubes and produce new sprouting capillary branches, or they become transformed into strands of common fibroblasts. This corresponds exactly with the facts I found 22 years ago in common aseptic inflammation—the proliferating endothelium of the capillary veins may produce fibroblasts but never gives rise to cells of ameboid, wandering character, to polyblasts. The epithelioid cells, as is well known, belong to the vast group of polyblasts.

Even in cases in which the fibroblasts of endothelial origin, under the chemical influence of the tubercle bacilli, assume an embryonic, mesenchymatous character, they remain distinctly separated from the epithelioid cells of reticular or lymphocytic origin and do not play any rôle in giant cell formation.

The conditions change completely if we widen the boundaries of the endothelium idea and apply the term "endothelium" to all squamous cells in general, lining blood or lymph-spaces of any kind, for instance to the cells of the wall of the venous sinuses of the spleen, to the cells of Kupffer in the liver, to the reticular cells lining the walls of the lymph node sinuses, etc. If we choose this point of view, we shall have to look on the epithelioid and giant cells of the tubercles in the organs

mentioned as products of endothelial transformation. It is well known that in the liver the tubercles are built up chiefly by the cells of Kupffer (Oppenheimer,[29] Evans, Bowman and Winternitz[17]), in the lymph nodes by the reticular cells of the follicles and the sinuses (Joest and Emshoff[16]). But it seems to me that this would be a misleading way and a misuse of the term "endothelium." The elements just mentioned are structurally and functionally quite different from ordinary endothelium in common capillaries, arteries, veins, heart and lymph vessels. Whereas the latter is closely related with, and, as we have seen, easily becomes transformed into fibroblasts, the squamous elements, lining the spaces mentioned above, show ontogenetically and physiologically close connections with the great family of wandering cells.

Scattered all over the body, the wandering cells, being derived, in the earliest stages of ontogenesis, from the mesenchyme,[18] appear in different modifications. Part of them remain in an active and motile condition (the wandering cells of connective tissue); others are transformed into fixed, resting, nonmotile elements, but keep their potencies of motility in a latent condition forever. To this last category of cells, potentially motile and ameboid but normally quiescent, belongs a series of elements described by various authors under different names.

Here we have first the resting wandering cells of Maximow[30] or the clasmatocytes of Ranvier in the common loose connective tissue; second, the "adventitial cells" of Marchand or the "rhagiocrine cells" of Renaut, identical with the resting wandering cells of Maximow, in the omentum; third, the reticular cells and the cells of the sinuses in the lymph nodes, in the spleen and the bone marrow; fourth the cells of Kupffer in the liver; finally perhaps also the lining cells of the blood capillaries in some other organs, for instance the suprarenals.

Under the influence of stimulations of various kinds, all these cells can be mobilized and then become transformed into motile cells, functioning as active phagocytes, storing vital dyes and various other colloidal substances. For this reason they have been given the name of resting wandering cells by Maximow. They are all endowed with surprisingly manifold potencies and are able to proliferate and to differentiate into various other forms. For emphasizing this quality, I have called them (1902) in their active, mobilized condition on the field of inflammation, polyblasts.

[29] Virchows Arch. f. path. Anat., 1908, 194 Beiheft, p. 254.
[30] Arch. f. Mikrosk. Anat., 1906, 67, p. 680.

Goldmann [15] and Tschaschin [31] were the first who clearly outlined the idea that they all belong to one large group and that they are endowed with identical physiologic properties and prospective potencies. Goldmann gave them the name of "pyrrhol cells." Kiyono [12] has grouped them together under the name of "histiocytes." Although this term means nothing else than "tissue cells," it seems to have been largely adopted by pathologists. Evans [32] gave them (in their mobilized condition) the name "macrophages," used in the old days by Metschnikoff.[5] By some other writers the whole group, scattered over the various organs and tissues, is referred to as the "reticulo-endothelial apparatus."

These resting wandering cells or histiocytes in all their modifications in the different organs and tissues—and not the common endothelial cells—are the most active elements in the various inflammatory processes and in particular in tuberculosis. Under the influence of the inflammatory stimulation they are mobilized and furnish polyblasts. When the inflammation subsides, the polyblasts not spent and destroyed in the course of the process, return to the resting condition. In the normal organism they again—and not the common endothelial cells—are probably the source of the so-called "endothelial leukocytes" or the monocytes of the blood.

It is natural that the lymphocytes should also play an active rôle in the same inflammatory processes and join the polyblasts of histiocytic origin. The study of the embryonic histogenesis of blood and connective tissue shows that they are closely related genetically with the histiocytes or resting wandering cells (Maximow,[33] Alfejew [34]). In the embryo many of them normally become quiescent in the later periods of intra-uterine life and are converted into resting wandering cells or histiocytes. The histiocytes, for instance the reticular cells of the blood forming organs, on the other hand, may produce lymphocytes, and this is true even for the adult organism. As I have recently shown, the reticular cells or histiocytes of the lymphoid tissue, under certain circumstances, may produce large lymphocytes even in tissue cultures.[24]

The tubercle bacilli act on the tissue cells—and chiefly on the resting wandering cells or histiocytes—by means of specific chemical substances, and this action is pronounced in tissue cultures in stages in which no visible disintegration of the bodies of the bacilli themselves can yet be found. The cells may be influenced at a distance without coming in direct touch with the bacilli; the behavior of the bacilli toward

[31] Folia haematologica, 1913, 17, p. 317.
[32] Am. Jour. Physiol., 1915, 37, p. 243.
[33] Folia haematologica, 1907, 4, p. 611.
[34] Ibid., in press.

the cells can therefore by no means be compared with the action of inert foreign bodies of the same size and consistency.

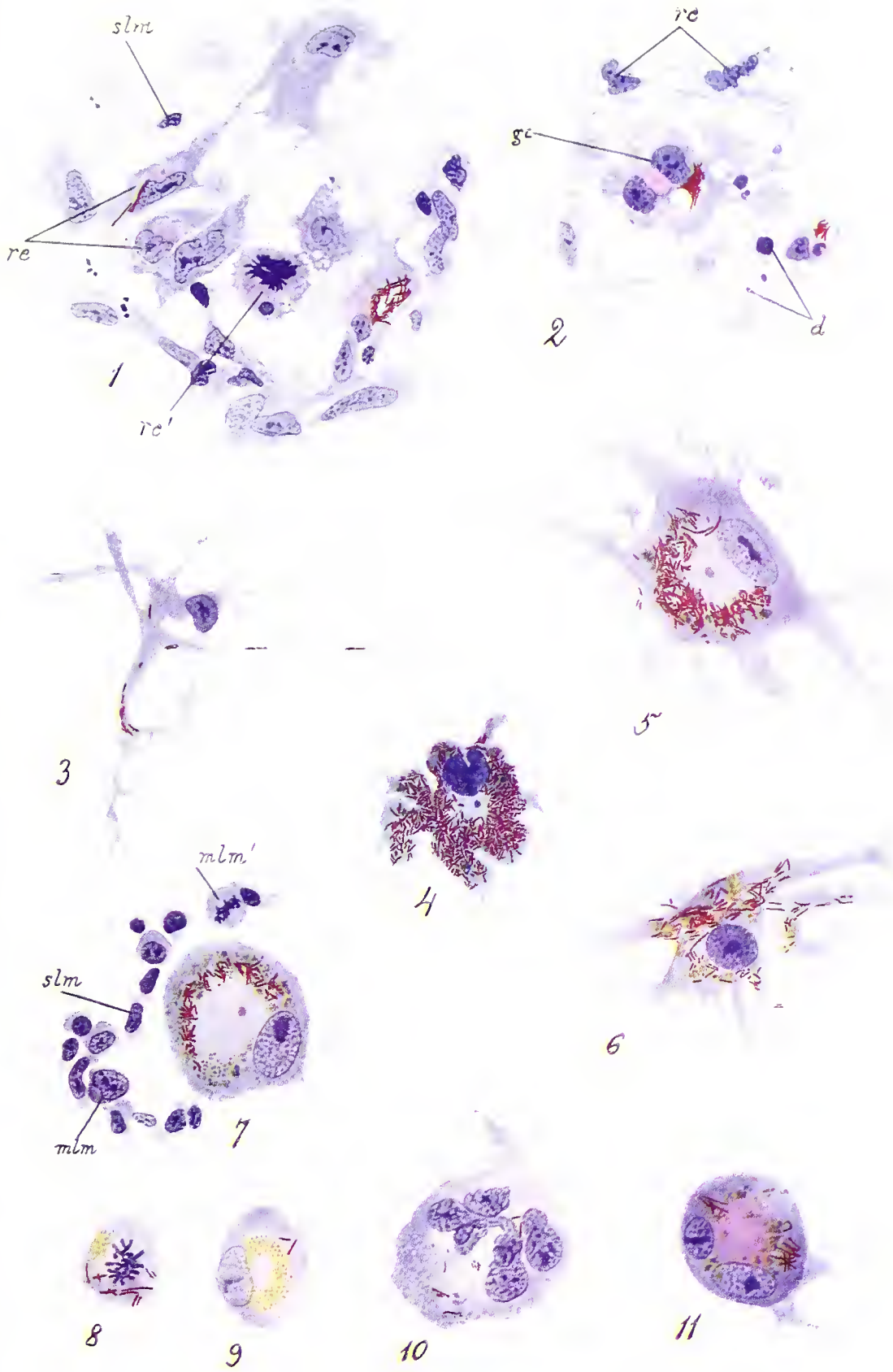
The specific chemical stimulation, originating in the tubercle bacilli, was thought by R. Virchow and Baumgarten to cause cell hypertrophy and proliferation directly, whereas Weigert's idea was that the first effect is an injury to the living substance of the cells, causing its degeneration, which is only secondarily followed by a reaction of progressive character.

The facts, observed in the inoculated cultures speak in favor of the first theory. The presence of a sufficient, sometimes very small, quantity of bacilli in a culture calls forth everywhere throughout the explant a distinct, peculiar change, chiefly in the resting wandering cells or histiocytes, manifesting itself in growth and hypertrophy, especially affecting the nucleus and the cytocentrum, and in changes of the surface tension conditions of the protoplasm; the immediate result may often be mitotic division, without a trace of previous degeneration.

Thus, the action of the tubercle bacilli is a direct specific formative stimulation, which might be compared, as Klebs did many years ago, to the action of the gall wasp on plant tissue. Of all the tissue elements, particularly the resting wandering cells or histiocytes are sensitive, as well as the lymphocytes, which join the former in their transformations. But the presence of the bacilli seems to have a general stimulating influence on the living substance of various other cells as well, causing, for instance, the fibroblasts and the true endothelial cells to remain for an abnormally long time in active condition or even to acquire embryonic properties, the fat cells rapidly to lose their fat, the small lymphocytes to hypertrophy and to transform themselves with exceptional ease into large lymphocytes, etc. Whereas, as I have recently shown (22, p. 521), in cultures of lymphoid tissue without bacilli, after the 6th day, usually a peculiar fibrillar intercellular substance develops between the fibroblasts of the new tissue, I never was able to find anything of this kind in the cultures inoculated with tubercle bacilli.

SUMMARY

The method of tissue culture enables us to duplicate the essential phases of the tuberculous process under artificial conditions. This makes it possible to follow the histogenesis of the lesions in living condition as well as in fixed and stained preparations in all its details and stages and to elucidate the origin of all the specific elements. It might be possible to use the same method for studying the biologic interrelations of the tissues and the bacilli under various conditions and influences.



All the figures represent sections of tissue cultures of lymphoid tissue, inoculated with tubercle bacilli of low virulence; in the case of fig. 19, bacilli of high virulence were used. Figure 23 on plate 6 represents a culture of omentum, inoculated with highly virulent bacilli; figures 24 and 25 represent cultures of common loose intermuscular connective tissue, inoculated with bacilli of low virulence. All figures have been drawn from slides, stained with carbol-fuchsin, hematoxylin and eosin-azure. A Zeiss achromatic immersion lens 1/12 and a compensating ocular 4 have been used.

c, indicates collagenous fibers; *d*, remnants of degenerated cells; *cp*, epithelioid cells; *fbl*, fibroblasts; *fble*, fibroblasts of endothelial origin; *fble'*, mitosis of an endothelial fibroblast; *fblem*, fibroblasts acquiring embryonic character; *gc*, giant cells; *lb*, lymphocytes containing tubercle bacilli; *llm*, large lymphocytes; *mlm*, medium lymphocytes; *mlm'*, mitoses of medium lymphocytes; *plb*, polyblasts; *rc*, reticular cells; *rc'*, mitoses of reticular cells; *slm*, small lymphocytes.

(Legends continued on next page)

Fig. 1.—Fixed reticular cells (*rc*) still attached to the fibers of the reticulum engulfing bacilli and beginning to hypertrophy and to divide (*rc'*). Five days in vitro, inoculation simultaneous with explanation.

Fig. 2.—Binucleated cell (young giant cell, *gc*) with a pink attraction sphere between the two nuclei. Same slide, as in fig. 1.

Fig. 3.—Polyblast of reticular origin with long, slender pseudopodia, containing bacilli; from the fibrin, surrounding the explant. Thirteen days in vitro (1 transplantation); inoculation simultaneous with explantation.

Fig. 4.—Ameboid reticular cells (polyblast or macrophage of epithelioid type) with massive pseudopodia; cell body crowded with bacilli and containing some green pigment granules; in the center a distinct attraction sphere with a group of darkly stained centrioles. Sixteen days in vitro (two transplantations); 10 days after inoculation.

Fig. 5.—Large ameboid reticular cell of the same type, with hypertrophied cytocentrum, bacilli and green pigment. Ten days in vitro (1 transplantation), 4 days after inoculation.

Fig. 6.—Large ameboid reticular cell of the same type with bacilli and their digestion products—gold yellow pigment granules. Nine days in vitro (1 transplantation), inoculation simultaneous with explantation.

Fig. 7.—Large spherical epithelioid reticular cell with hypertrophied sphere and centrioles, and bacilli and green pigment in the peripheral basophilic protoplasm zone. Around the cell clustering, proliferating (*mlm'*) small (*slm*) and medium (*mlm*) lymphocytes. Nine days in vitro (1 transplantation), 4 days after inoculation.

Fig. 8.—Mitosis of an ameboid reticular cell with bacilli, fat drops (vacuoles) and lipochrome inclusions (yellow granules). Twelve days in vitro (2 transplantations); inoculation simultaneous with explantation.

Fig. 9.—Similar cell in resting condition; sphere surrounded by thick layer of lipochrome granules. Thirteen days in vitro (2 transplantations), inoculation simultaneous with explantation.

Fig. 10.—Amitosis of nucleus in epithelioid reticular cell. Ten days in vitro (1 transplantation), 4 days after inoculation.

Fig. 11.—Epithelioid cell with two nuclei—transitional form to giant cells. Same slide as in Fig. 10.

PLATE 2

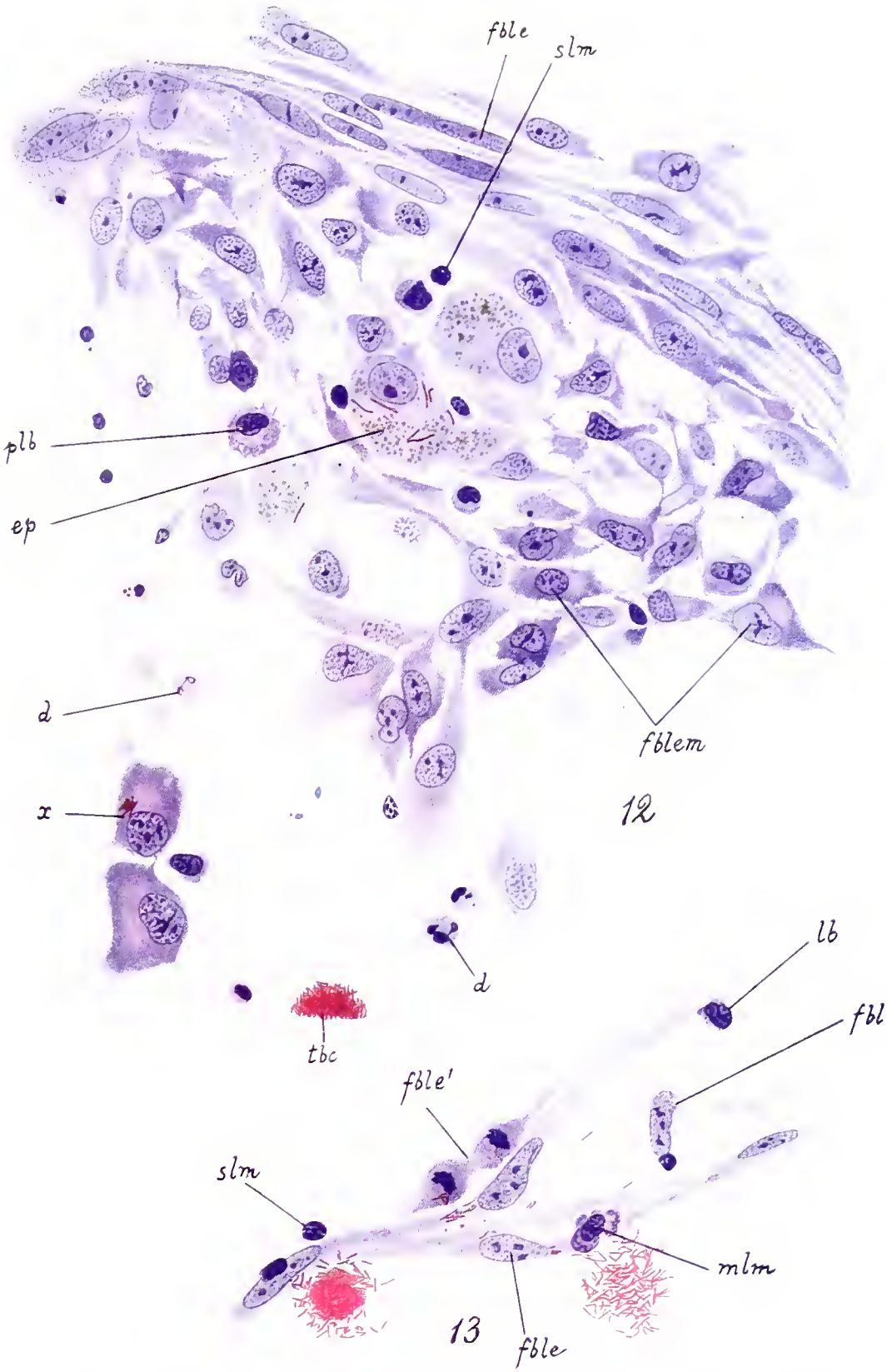


Fig. 12.—Proliferating fibroblasts (*fble*), arising from endothelium of an artery (not seen in the figure) and acquiring embryonic character (*fblem*); between them large epithelioid cells of reticular origin, with bacilli and green pigment (*ep*) and lymphocytes (*slm*), transforming themselves into polyblasts (*plb*); *x*, embryonic fibroblast, containing bacilli; *tbc*, edge of a colony of tubercle bacilli. Nine days in vitro (1 transplantation), 4 days after inoculation.

Fig. 13.—Strands of fibroblasts of endothelial origin (*fble*) containing bacilli and dividing (*fble'*); *lb*, lymphocyte, containing a tubercle bacillus. Ten days in vitro (1 transplantation), 4 days after inoculation.

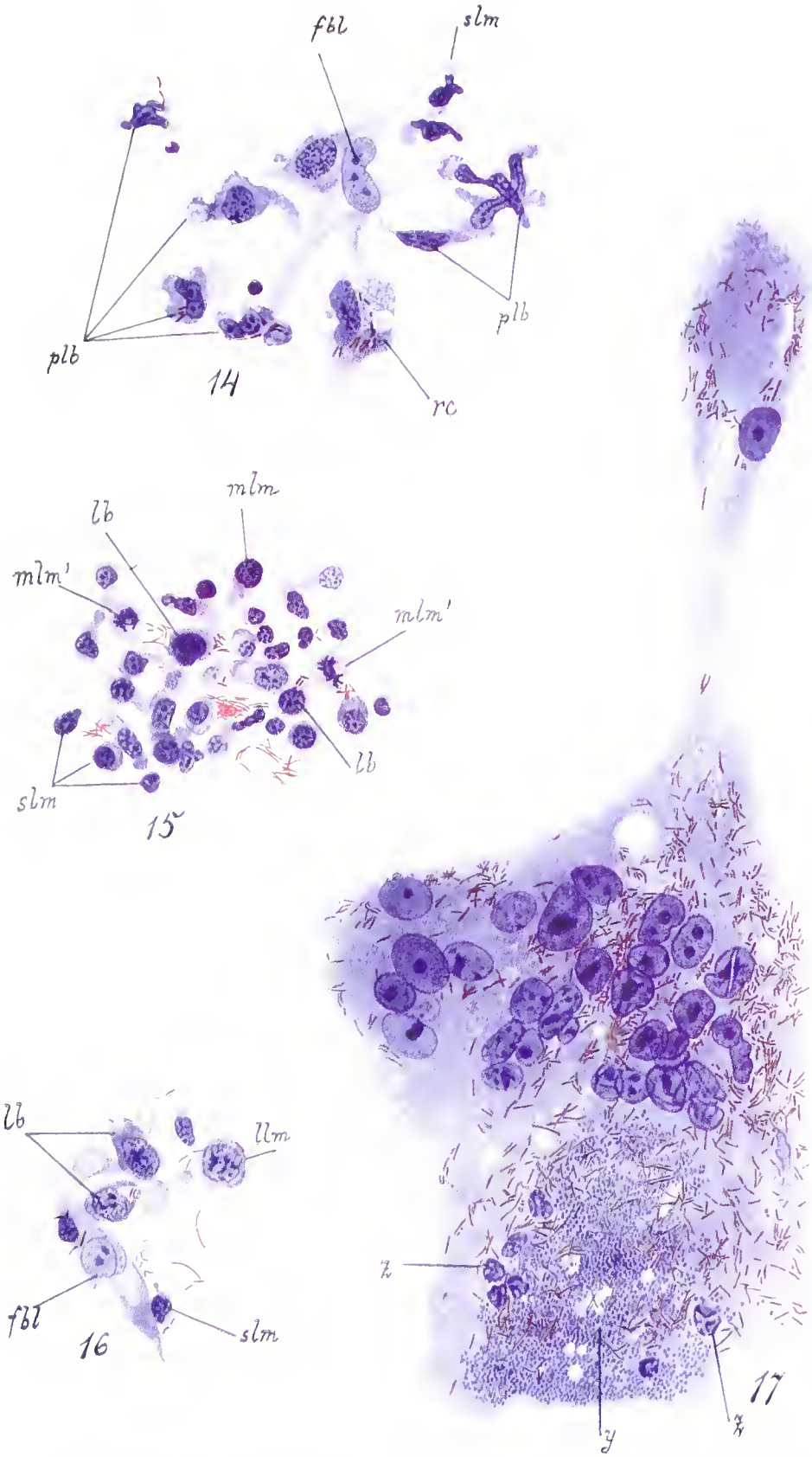
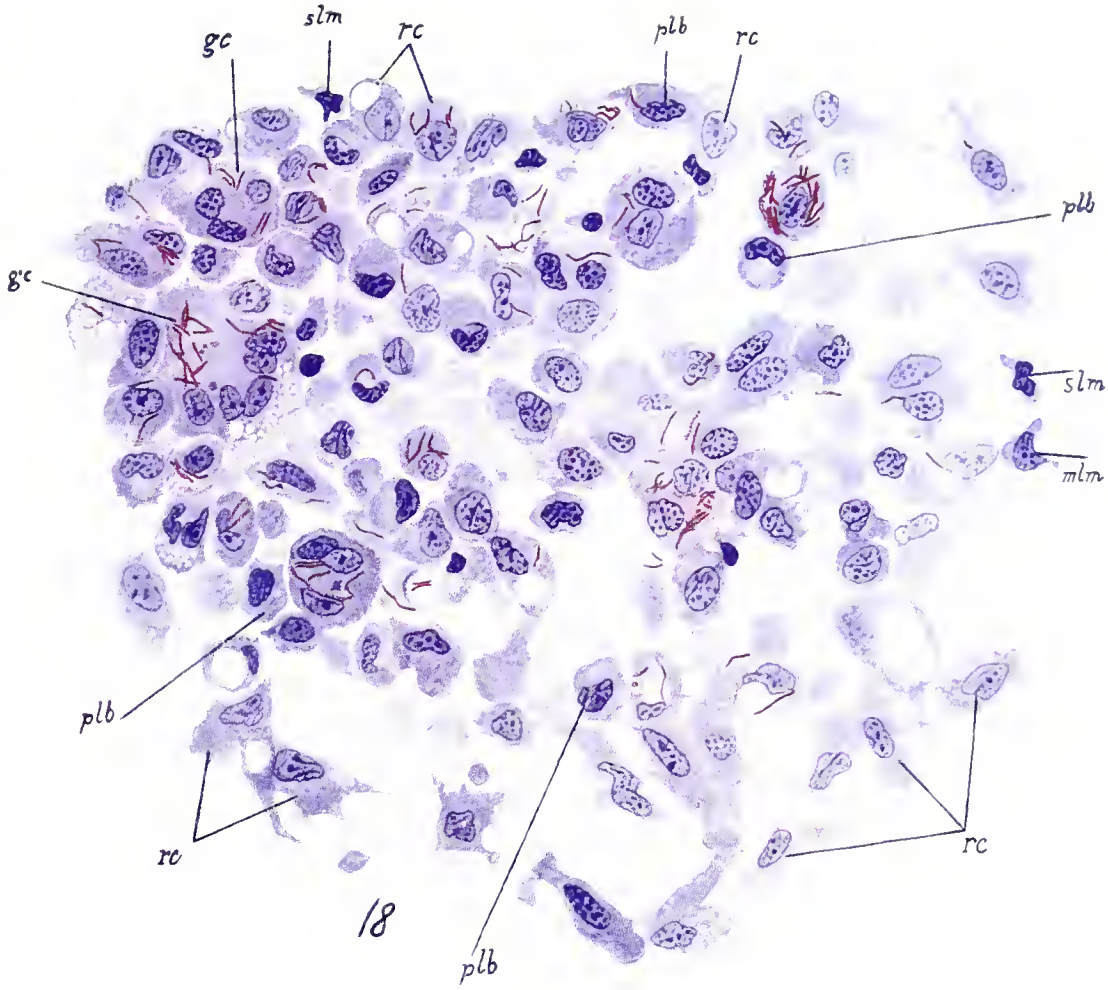


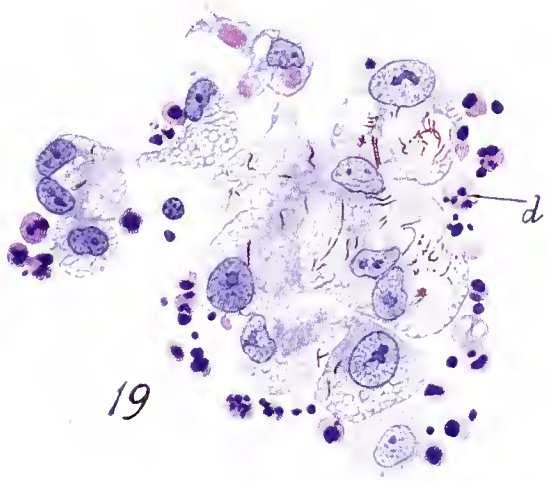
Fig. 14.—Group of ameboid medium sized (*mlm*) and small (*slm*) lymphocytes transforming themselves into polyblasts (*plb*), phagocytizing bacilli; *rc*, polyblast of reticular origin. Twelve days in vitro (2 transplantations), inoculation simultaneous with explanation.

Fig. 15.—Cluster of ameboid lymphocytes, small (*slm*) and medium sized (*mlm*), some of them dividing (*mlm'*), some containing bacilli (*lb*); from the layer of liquid, covering the explant. Ten days in vitro (1 transplantation), 4 days after inoculation.

Fig. 16.—Group of small (*slm*) and large (*llm*) lymphocytes, some of them (*lb*) containing bacilli. Same slide as in fig. 15.

Fig. 17.—Large multinucleated giant cell, containing numerous tubercle bacilli; in *y* saprophytic cocci in the protoplasm; *z*, degenerated nuclei. Fourteen days in vitro (3 transplantations), 10 days after inoculation.





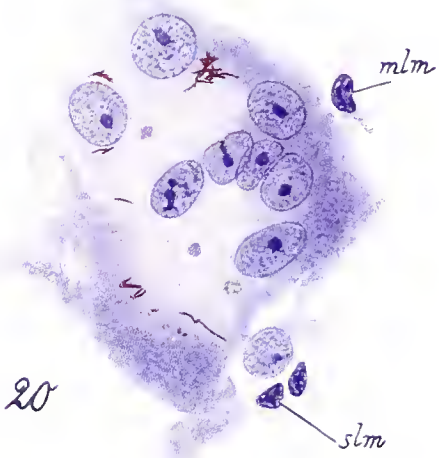
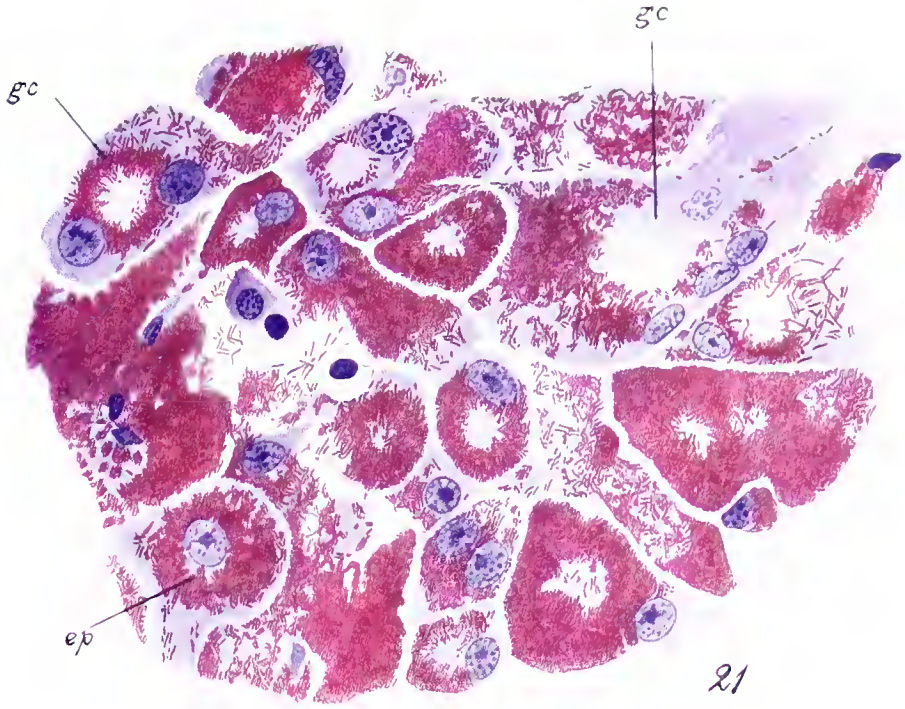


Fig. 18.—Large cell cluster (tubercle) from the layer of liquid covering the explant. Most cells, with clear nuclei (*rc*), are mobilized, ameboid, reticular cells, which have lost their green pigment and through proliferation became small. They contain bacilli and in *gc* fuse to multinucleated giant cells. Between them many cells with darker nuclei are seen—lymphocytes (*slm* and *mlm*) in the process of transformation into polyblasts (*plb*), joining the reticular cells in their further development and especially in giant cell formation. Nine days in vitro (1 transplantation), 4 days after inoculation.

Fig. 19.—Giant cell formation by fusion of epithelioid cells containing virulent bacilli and fat drops. Six days in vitro; inoculation simultaneous with explantation.

Fig. 20.—Typical giant cell of Langhans type; in the peripheral, blue protoplasm, surrounding the two central pink spheres with the groups of centrioles, bacilli and some few green pigment granules are seen—remnants of the reticular cell pigment. Ten days in vitro (1 transplantation), 4 days after inoculation.



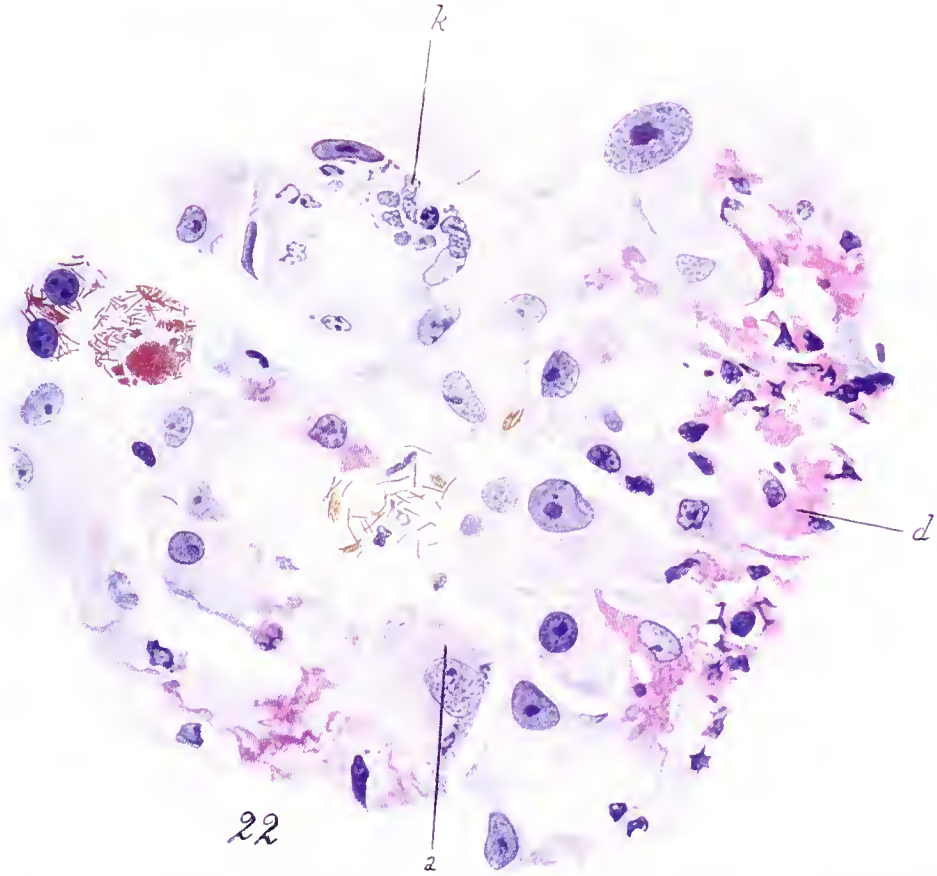


Fig. 21.—Part of a large tubercle consisting of mononucleated (*ep*) and multinucleated (*gc*) epithelioid cells, crowded full with tubercle bacilli. Eight days in vitro (1 transplantation); inoculation simultaneous with explantation.

Fig. 22.—Caseous degeneration of a tubercle. Most of the epithelioid and giant cells show karyorrhexis and karyolysis (*k*); the protoplasm is peculiarly pale, either homogeneous or finely alveolar (fat droplets) and contains lumps of an acidophilic substance (*a*). The central cell contains tubercle bacilli and their degeneration products—yellow pigment. Same slide as in fig. 21.

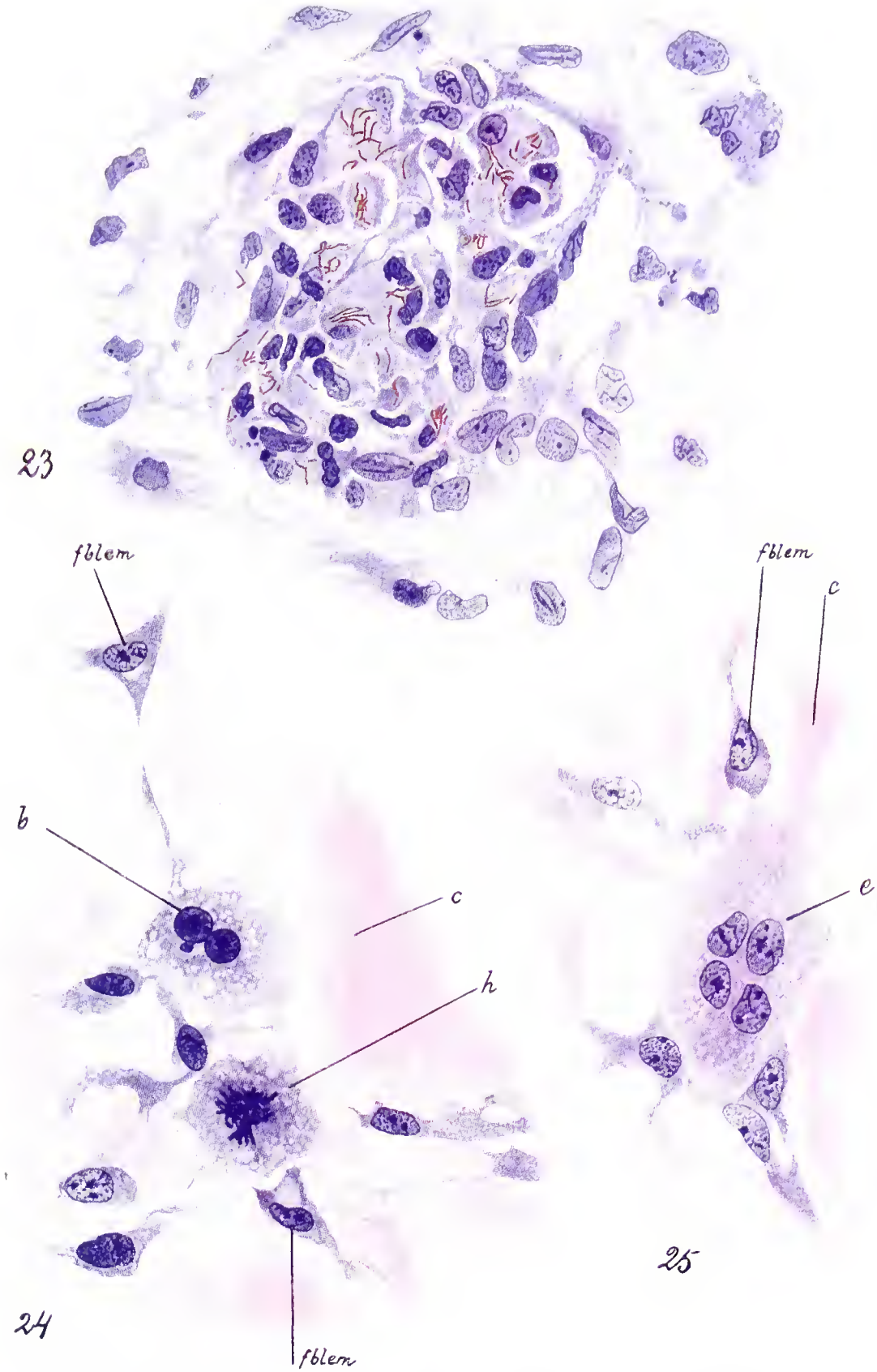


Fig. 23.—Tubercle in the tissue of the omentum. Ten days in vitro, inoculation simultaneous with explantation.

Fig. 24.—Changes of the common loose intermuscular connective tissue induced by tubercle bacilli; *fblem*, fibroblasts, acquiring embryonic character; *b*, hypertrophied resting wandering cell with amitosis of nucleus; *d*, similar cell with mitosis. Nine days in vitro (1 transplantation); inoculation simultaneous with explantation.

Fig. 25.—Same tissue; small giant resting wandering cell with four nuclei (*e*), surrounded by embryonic fibroblasts (*fblem*). Same slide as in fig. 24.

MEDIUM FOR INHIBITION OF SPREADERS AND DIFFERENTIATION OF B. COLI AND B. AEROGENES

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INTRODUCTION

Many mediums have been used for the isolation and identification of the colon bacilli [1] following preliminary enrichment in lactose broth. In the United States, litmus lactose agar and Endo medium have been largely used. On the European continent, the Drigalski-Conradi agar [2] has been more often employed, while in England, the MacConkey bile agar [3] has also been used.

Besides these, other solid carbohydrate agar mediums are used locally to a limited extent, among which might be mentioned litmus gentian violet agar,[4] brom cresol purple agar, litmus mannite agar, and neutral red agar. These mediums are all peptone-sugar agars to which appropriate indicators have been added. In a few cases an agent has been included which inhibits the development of some of the other bacteria likely to be encountered in routine water analysis.

There are many objections to the mediums mentioned.

1. The acid formed from the colon bacilli (acid and aldehyde in the case of the Endo agar) diffuses rapidly, and colonies close together cannot be distinguished, the colonies of the colon bacilli often appearing the same as the nonlactose splitters. Frequently the whole plate is discolored, and it is somewhat difficult to differentiate the colonies with any degree of accuracy.

2. Lactose fermenting organisms of the coli-aerogenes group produce alkali after the acid fermentation has taken place. The acids which are formed from the lactose are further attacked and split into neutral or alkaline salts, the acid color of the medium changing thereby to the alkaline color.[5] This is especially true with B. aerogenes.

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[1] All gram-negative nonspore-forming rods capable of fermenting lactose with the evolution of gas are included in the term colon bacilli. Bact. coli refers to those colon bacilli which do not give the Voges-Proskauer reaction, the genus Escherichia as defined by Bergey (Manual of Determinative Bacteriology, 1923). These colon bacilli which give a positive Voges-Proskauer test are included in this paper under the term Bacterium aerogenes (Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 20). Bergey divides B. aerogenes into 6 species of the genus Aerobacter.

[2] Ibid., 1902, 39, p. 283.

[3] Thompson-Yates Laboratory Reports, 1900, 3, p. 151.

[4] Hall and Ellefson: Jour. Bacteriol., 1918, 3, p. 329.

[5] Ayers, S. H., and Rupp, P.: Jour. Infect. Dis., 1918, 23, p. 188.

3. Contaminating alkali-forming organisms often produce enough ammonia or other basic substances to neutralize the acid produced by the colon bacilli, as soon as it is formed, so that, although B. coli is present, no acid zone is seen around the colony.

4. Many of these ammonia-producing organisms are also "spreaders," which ofter grow so rapidly as to cover the plate in a short time, thereby preventing the isolation or identification of B. coli or B. aerogenes.

Growth of these ammonifiers and "spreaders" may often be inhibited by the addition of bile,[3] phenol [6] or some inhibitory dye to the medium, as in the Drigalski-Conradi agar. Such dyes, however, do not prevent the growth of gram-negative organisms, among which are Bact. alkaligenes and Bact. fluorescens and other strong alkali producers often developing on plates streaked from lactose broth tubes that have been inoculated with water. The use of bile as an inhibitory agent has not found favor in the United States,[7] because it has been shown that many strains of B. coli itself are inhibited by bile.[8]

Another solid medium for the isolation of B. coli has recently been suggested. This is a modification of the Holt-Harris and Teague [9] eosin-methylene blue agar, much simplified by Levine.[10] As made by him, this medium is perhaps the easiest and simplest to prepare of all the confirmatory agars now in use. The latest standards of the American Public Health Association [11] have designated this medium as one of the "standard media" for use in water analysis. It is a pepton-phosphate lactose agar to which are added eosin and methylene blue solutions. The medium is unadjusted, and since it is opaque, filtering is unnecessary. The colony of B. coli has a characteristic color and appearance on this medium, as has that of B. aerogenes, and the one may be distinguished from the other in about 90% of the cases. Instead of the organisms being distinguished by the zones around the colonies, the colony itself has the characteristic color. Thus, although a colony of B. coli is surrounded by nonlactose fermenters, or even alkali producers, the colony may be easily distinguished. In addition, the nonfecal

[6] Thompson-Yates Laboratory Reports, 1900, 3, p. 1. Cited by Prescott and Winslow. Elements of Water Bacteriology, 1913.

[7] Levine: Iowa State Coll. Eng. Exper. Sta., 1921, Bull. 62.

[8] Jordan, E. O.: Jour. Infect. Dis., 1913, 12, p. 326.

[9] Ibid., 1916, 18, p. 596.

[10] Ibid., 1918, 23, p. 43.

[11] Standard Methods for Water Analysis, 1923.

organisms of the B. aerogenes type may be distinguished at least presumptively from the true B. coli which is of so much more sanitary importance.

Although this medium offers so many advantages, it was found by one of us to be inferior to the Drigalski-Conradi and to the Hall and Ellefsen gentian violet mediums [4] in one respect, namely, that it did not prevent the growth of "spreaders," because no inhibitory dye was used. Both eosin and methylene blue exert very weak bactericidal properties, if pure compounds are used.

In routine analysis of muddy waters and in research work with soils, the spreaders of the B. mycoides and B. cereus type are particularly troublesome. If the combination of an inhibitory dye with the standard eosin methylene blue agar would inhibit the spreaders and still show the advantages of that medium, we should have a medium much better suited for routine or research work for the isolation and identification of B. coli than any we now have.

The possibility of using an inhibitory dye, and thus inhibiting the spreaders, with the eosin methylene blue agar at once suggested the possibility that the colon bacilli themselves might not be easily distinguished on such a dark colored medium, or that they might not show typical colonies. Also in view of the fact that Churchman [12] has reported some strains of B. coli to be gentian violet sensitive, it seemed advisable to test a large number of organisms on mediums varying the amount of dye used, and thus determine whether or not the organisms of the colon group isolated from water are likely to be dye resistant. Likewise, it was thought advisable to test the effect of the dye on spreaders, and to find a concentration of dye in which all the colon bacilli would survive but the spreaders be inhibited. The use of dye in the enrichment broth, following the method of Hall and Ellefsen,[4] was found inapplicable for muddy waters or soil, for the colloidal matter had the effect of precipitating the dye and the gram-positive organisms were little inhibited. Stronger concentrations of dye could not be used for the reasons enumerated by Hall and Ellefsen.[13]

EXPERIMENTAL

Effects of Various Concentrations of Crystal Violet in Eosin-Methylene Blue Agar on Pure Cultures of B. coli and B. aerogenes.—Seventy-three strains of B. coli and 27 of B. aerogenes were isolated from a grossly polluted stream, Delaware and Raritan Canal, and from soil. Levine's eosin methylene blue

[12] Jour. Exper. Med., 1921, 33, p. 569.
[13] Jour. Am. Water Works Assn., 1919, 6, p. 67.

agar and litmus lactose agar were used for the isolation so as to avoid any chance that the strains isolated might be selected resistant to triphenylmethane dyes. B. coli and B. aerogenes were separated on the basis of the Voges-Proskauer reaction. The 100 strains were inoculated into lactose broth and later were streaked on 4 plates, one being the unmodified standard eosin methylene blue agar, and the other 3 having different percentages of crystal violet. The results are shown in table 1.

TABLE 1

EFFECTS OF DIFFERENT CONCENTRATIONS OF CRYSTAL VIOLET ON THE COLONY GROWTH OF B. COLI AND B. AEROGENES

Dilution of Crystal Violet	Appearance of Growth in 24 Hours
Check, no dye	Typical colonies
1:10,000	Small, restricted colonies
1:20,000	71 restricted atypical colonies; 29 typical colonies
1:100,000	All colonies large and typical, composing favorably with check

The colony formation of B. coli and B. aerogenes on the medium containing 1:100,000 crystal violet differs slightly from that on the unmodified eosin methylene blue agar, but is at least as typical as on that medium. The description of typical colonies of B. coli and B. aerogenes in 24 hours on this medium is as follows: B. coli colonies black, or dark bluish violet by transmitted or reflected light, having a decided tendency to show a greenish metallic sheen, showing little tendency to run together, with a dark center reaching to the edge or nearly to the edge of the colonies; B. aerogenes colonies, usually sticky, showing a decided tendency to run together, pink or light lavender by reflected light, by transmitted light usually dark at the center and light at the edges, having no tendency to show a metallic sheen, dark center of colonies reaching not more than half way to the edge; contaminating colonies, usually pink or light colored, usually with no dark centers, or if with dark centers, blue not black. Both B. coli and B. aerogenes are more typical in 48 than in 24 hours.

The medium as modified by the authors will be called triple dye agar and is simply the standard eosin methylene blue agar of Levine [7, 10, 11] to which 1 c c. of a 0.1% (aqueous) solution of crystal violet is added per 100 c c. of medium at the time the other dyes are added.[14]

[14] Crystal violet, hexamethyl-pararosanalin chloride, is a pure compound and has the same selective bactericidal action as does gentian violet. Gentian violet is a trade name; this stain is a mixture of dyes of which crystal violet is one, and it varies as manufactured by different firms. For this reason it is advisable to replace gentian violet with crystal violet, both for use in the Gram stain and in medium (Conn, H. J.: Jour. Bacteriol., 1922, 7, p. 529). Methylene blue comes in two forms—the alcoholic soluble double zinc salt and the water soluble pure compound. The former compound is somewhat bactericidal, the latter much less so. The zinc-free compound is known as "medicinally pure methylene blue" and can be purchased from most dye manufacturers or from a drug store. Many of the "methylene blue for bacilli" including the prewar Grübler compound were mixtures of the two compounds (Wall, W. A., and Robertson, A. H.: Ibid., p. 307). The medicinally pure compound should be used in medium, for obvious reasons.

GROWTH OF SPREADERS ON TRIPLE DYE AGAR

Fifty spreaders were isolated from the soil. No attempt was made to identify them, although it was noticed that many of them resembled B. mycoides and B. cereus in colony formation and microscopic appearance. They were streaked on the triple dye agar and the growth observed. None of the 50 organisms showed growth in 24 hours; in 48 hours, 21 developed slightly, and the other 29 showed no visible growth.

These two experiments demonstrated that crystal violet dye in the concentration of 1:100,000 in the triple dye agar sufficiently inhibited the development of gram-positive spreaders, but showed no deleterious effects on the growth of 100 strains of the colon bacilli.

Efficacy of the Triple Dye Agar as a Confirmatory Medium for the Colon Bacilli and as a Differential Medium for B. coli and B. aerogenes.—Plates of triple dye agar were streaked from lactose broth enrichment cultures or were streaked directly from the following sources: Delaware-Raritan Canal, soil, feces and liquid from trickling filtration beds of a sewage disposal plant. In most cases plates of litmus lactose agar, Endo agar, and standard eosin methylene blue agar were streaked for comparison. From these plates, after 24 hours' incubation, colonies of B. coli and B. aerogenes were picked (not more than one of each species from a plate) and inoculated back into lactose broth for the "completed" test, and also into dextrose broth for the Voges-Proskauer test. This test was used to confirm the accuracy with which B. coli could be differentiated from B. aerogenes on the triple dye agar, as is shown in the following table.

In the comparison with the growth on the Endo or litmus lactose agar, the colonies on the triple dye agar showed up much plainer with both organisms, particularly when soil and water were plated. On only 3 were spreaders developing, and their growth appeared to be enhanced by the moist condition of the agar. In most cases, the colonies of B. coli and B. aerogenes were distinct and easily differentiated from the noncolon forms and from each other. In many cases isolations were made from the triple dye agar, while on the litmus lactose and Endo agar plates, no typical colonies could be found. In comparison with the standard eosin methylene blue agar, the triple dye agar showed a distinct advantage in that the "spreaders" were inhibited. In many of the plates of the unmodified standard eosin methylene blue agar, particularly those streaked from soil, spreaders had covered all the colonies and the isolation of the colon bacilli was next to impossible. Isolations were readily made, however, from the plates of the triple dye agar.

It will be seen from table 2 that differentiation of B. coli from B. aerogenes from colony formation on the triple dye agar is fairly accurate. Ninety-four % of the colonies called B. coli and 93% of

the colonies called B. aerogenes were correctly established as determined by the Voges-Proskauer reaction.

It is also worthy of note that of the 90 strains isolated from feces, only one was B. aerogenes (1.12%), while of the 63 strains isolated from soil, 57 were B. aerogenes (90.47%). This would confirm the work of Johnson and Levine,[15] Chen and Rettger,[16] Rogers, Clark and Lubs,[17] and others, that the colon bacilli of the soil are largely B. aerogenes while those of the intestinal tract are largely B. coli and emphasizes the need of distinguishing these two in water analysis. According

TABLE 2

ACCURACY OF DIFFERENTIATION OF B. COLI AND B. AEROGENES ON TRIPLE DYE AGAR

Source of Culture	Treatment	No. of Colonies Isolated Called B. coli	No. of Colonies Called B. coli Which Were Actually B. coli	No. of Colonies Isolated Which Were Called B. aerogenes	No. of Colonies Which Were Actually B. aerogenes
Delaware-Raritan Canal...	Preliminary enrichment in lactose broth	59	58	22	20
Delaware-Raritan Canal...	Preliminary enrichment in lactose broth	17	17	3	3
Soil.....	Preliminary enrichment in lactose broth	6	4	28	28
Soil.....	Preliminary enrichment in lactose broth	9	2	20	20
Feces.....	Preliminary enrichment in lactose broth	30	29	0	0
Feces.....	Streaked direct without enrichment	25	25	0	0
Feces.....	Streaked direct without enrichment	55	35	0	0
Sewage.....	Streaked direct without enrichment	20	19	6	6
Sewage.....	Preliminary enrichment	34	33	20	15
Total.....	.....	235	222	99	92

Percentage B. coli correct = 94.4%. Percentage B. aerogenes correct = 92.99%.

to our present A. P. H. A. Standards,[11] the presence of B. aerogenes condemns water as much as does B. coli (so-called "completed test for B. coli"), in spite of the accumulating evidence that B. aerogenes is not primarily a fecal organism.

SUMMARY

The addition of 1:100,000 crystal violet to the A. P. H. A. standard eosin-methylene blue agar inhibits the development of spreading colonies.

[15] Jour. Bacteriol., 1917, 2, p. 379.
[16] Jour. Bacteriol., 1920, 5, p. 253.
[17] Ibid., 1918, 3, p. 231.

The addition of this amount of dye to the standard agar does not make the identification or isolation of the colon bacilli more difficult. On the contrary, the colonies are even more typical than on the standard agar.

B. coli and B. aerogenes can be fairly well distinguished from each other by colony appearance on this agar.

B. coli predominates in feces, and B. aerogenes in soil.

DESTRUCTION OF URIC ACID BY BACTERIA AND MOLDS

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Observations by one of us after ingestion of uric acid indicated destruction of considerable quantities of that substance in the alimentary tract. After ingestion of 1 gm. of uric acid only one-fourth of that amount was present in the feces. Analyses of blood and urine gave no evidence that the missing quantity had been absorbed. Similar results were secured on feeding uric acid to a dog.

It is well known that solutions of uric acid for analytical work require addition of preservatives to prevent deterioration. Chloroform is added to the usual phosphate-acetate uric acid standard for this purpose. Recalling this rather surprising demonstration of adaptation of micro-organisms to a very poor medium, feces were added to the uric acid standard in an attempt to determine whether there were organisms present which were capable of destroying this purine under such conditions. The results of analyses made throughout a period of days appear in table 1. The slow decrease in uric acid content during the first day or two, followed by greater destruction as well as the absence of destruction in the presence of chloroform, suggests the utilization of the uric acid by micro-organisms.

Little seems to be known of the micro-organisms having the capacity of using uric acid or other purines. The work of Baginski[1] and Schindler[2] many years ago indicated slow but rather complete destruction of purines by intestinal bacteria. Schittenhelm and Schröter[3] concluded from their results that elementary nitrogen is a product of destruction of the purine portion of yeast nucleic acid by intestinal bacteria. The work of Oppenheimer[4] and later of Sivèn[5] verified purine destruction but left the identity of the nitrogen containing product in doubt. Thannhauser and Dorfmüller[6] showed conclusively that the purine ring structure of guanosine, adenosine and inosine is disrupted by a mixture of intestinal bacteria, with ammonia the main nitrogenous product. These investigators did not study utilization by micro-organisms of the nitrogen contained in free purines. So far as we are aware the extent of investigations of the purines in this connection, has been rather limited.

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[1] Ztschr. f. physiol. Chem., 1884, 8, p. 395.
[2] Ibid., 1889, 13, p. 441.
[3] Ibid., 1904, 40, p. 62; 1904, 41, p. 284.
[4] Ibid., p. 3.
[5] Pflüger's Arch., 1914, 157, p. 582.
[6] Ztschr. f. physiol. Chem., 1918, 102, p. 148.

Sestini[7] and Gerard[8] were the first to show that uric acid is destroyed by micro-organisms. According to their results, urea is the true product of uric acid decomposition, and ammonium carbonate results from the action of a urophagic microbe on urea. These authors did not use pure cultures. Later Ulpiani[9] isolated an encapsulated cocco bacillus from chicken excreta which was capable of growth in a uric acid medium. He described the organism as pigmented, gram-positive and readily stained by Ziehl and also by gentian violet. On agar its colonies were elevated and appeared yellowish, attaining a diameter of 1 to 1.5 mm. at the end of 3 days. The margin of the colony was sharp and smooth, its center dark. A homogeneous mass of yellowish granules was observed. Growth on gelatin was the same as on agar. Broth became uniformly cloudy at the end of 24 hours with the formation of a thin film. No film was formed in peptone water. Ulpiani made all his studies by means of the qualitative murexide reaction. He further found that Bacterium ureae does not attack uric acid. Similar experiments were made by Cingolani[10] who attributed the uric acid destruction to reactions of hydrolysis and oxidation. So far we have not found any further reference to this organism in bacteriologic

TABLE 1

DESTRUCTION OF URIC ACID IN A FECES INFUSION

Days of Growth	200 C c. Standard*	200 C c. Standard* +2 C c. $CHCl_3$	200 C c. Standard* +2 C c. Feces	200 C c. Standard* +2 C c. Feces +2 C c. $CHCl_3$
Immediate	98%	101%	98%	101%
2 days	103%	100%	95%	95%
3 days	98%	101%	72%	97%
5 days	97%	100%	26%	93%

* Phosphate uric acid standard, containing 1.4 gm. glacial acetic acid, 9 gm. Na_2HPO_4, 1 gm. NaH_2PO_4, and 200 mg. uric acid per liter.

literature. Liebert[11] found that uric acid was oxidized by B. pyocyaneus, B. fluorescens (liq. and non liq.), B. stutzeri and a spore-bearing anaerobe. Carbon dioxide and ammonia were produced and as intermediate products, allantoin, urea and oxalic acid. Kossowicz[12] reported 10 species of molds capable of utilizing uric acid, hippuric acid and glycine as sources of carbon and nitrogen. He believed that an enzyme was involved in the destruction of uric acid and that it was distinct from another which decomposed hippuric acid. McDermott[13] found C. glaber and C. pfefferianus capable of utilizing uric acid, hippuric acid and urea but not hexamethylenetetramine. Recently Koser[14] reported a uric acid medium by use of which it was possible to differentiate between B. coli and B. aerogenes. In this medium, containing NaCL, $MgSO_4$, $CaCl_2$, K_2HPO_4, glycerol and uric acid, B. aerogenes grew luxuriantly while B. coli failed to develop. No report was made of corresponding destruction of uric acid. We have found 6 strains of B. aerogenes to grow and utilize uric acid in Koser's medium.

[7] Gazzeta Chimica., 1896, 26, p. 92.
[8] Compt. rend. Soc. de biol., 1896, 123, p. 185.
[9] Gazzeta Chimica., 1903, 33, p. 93.
[10] Ibid., p. 98.
[11] Chemical Abstracts, 1911, 5, p. 2398.
[12] Ibid., 1913, 7, p. 807.
[13] Ibid., 1914, 8, p. 514.
[14] Jour. Infect. Dis., 1918, 23, p. 377

In our own studies we have endeavored to find bacteria capable of destroying uric acid in the simplest possible medium—uric acid standard solutions such as are used in quantitative work. The ones chosen were the phosphate-acetate standard described by Benedict and Hitchcock [15] (containing 9 gm. Na_2HPO_4, 12 H_2O; 1 gm. NaH_2PO_4, H_2O; 1.4 c c. glacial acetic acid and 200 mg. uric acid per liter) and the same solution without addition of the acetic acid, having $p_H = 6\pm$ and $7\pm$ respectively. We were primarily interested in organisms found in cultures from human feces, but other bacteria were also investigated. We also isolated the B. acidi-urici of Ulpiani, confirmed its capacity for destruction of uric acid, and endeavored to determine whether it possesses uricase. Finally, we observed a series of pathogenic and nonpathogenic molds to determine their capacity for growth with uric acid as the source of nitrogen. All observations were quantitative, determinations being carried out colorimetrically with the Morris and Macleod [16] technic (preliminary precipitation as zinc urate being omitted).

Maintaining the simplest possible medium has been of primary importance in our first search for organisms capable of growth on the uric acid. The number of organisms growing under this condition is necessarily small. We plan to increase it with such other organisms as can utilize uric acid under stimulation from added substances. With this idea we have added glucose, glycerol, asparagine and Witte's peptone to the uric acid standards. In general, we can state the result of such attempts as follows: Though asparagine and Witte's peptone encourage growth, both supply nitrogen in more readily available form than uric acid, and the latter is not utilized. Glucose and glycerol stimulate growth, and their presence is thus a contributory factor in the utilization of uric acid. It is well in this connection to note that in sterilization of the phosphate mediums containing glucose there is some conversion of glucose to more reactive forms, which will reduce the arseno-tungstate complex of the uric acid reagent. As a result the blue color by which uric acid is determined is increased, thus increasing the apparent value of uric acid present in the same solution or tending to balance any decrease in uric acid resulting from organisms under observation. Fortunately the extent of this error due to glucose has not exceeded 10% and is thus of minor importance in determination of those organisms which utilize uric acid.

[15] Jour. Biol. Chem., 1915, 20, p. 619.
[16] Ibid., 1922, 50, p. 55.

There are probably many other conditions which influence the speed of destruction of uric acid by organisms or indeed determine whether utilization can take place. One of these is apparent even in using the two slightly different uric acid standards previously described. We have found that many of the organisms behave differently in the two solutions. Table 2 shows the markedly greater speed of uric acid destruction observed in a feces infusion of the phosphate solution without acetic acid. Results presented in later tables indicate similar effects on pure cultures. Without doubt the addition to the mediums of such ions as chloride, sulphate, magnesium and calcium would exert an even greater influence on growth. This would add to the list of those which utilize uric acid. The present communication is the report of attempts to discover which of the forms capable of growing on the simplest possible mediums will utilize uric acid.

TABLE 2

EFFECT OF MEDIUM VARIATIONS ON THE DESTRUCTION OF URIC ACID IN A FECES INFUSION

Days of Growth	Phosphate Uric Acid Standard With Acetic Acid	Phosphate Uric Acid Standard Without Acetic Acid
1 day	88%	94%
3 days	90%	16%
5 days	70%	0

Of several micro-organisms found in fowl excreta, the pure cultures of only two destroyed uric acid under these conditions. One of these (undetermined) did so in a definite but limited way; the other destroyed it completely in 2 or 3 days and proved to be identical with the uric acid bacterium of Ulpiani. Since the colorimetric method of analysis permits accurate quantitative determinations of smaller amounts of uric acid than could be qualitatively tested by the murexide reactions used by Ulpiani, it was desirable to follow the course of uric acid destruction. Sets of test tubes of standard uric acid were inocculated with the organism and these analyzed so as to give readings at intervals of 6 hours throughout 2 days. In table 3 are recorded the results of such a series. It will be noted that there was no destruction of uric acid during the first 6 hours, and that the destruction was variable in rate throughout the following 30 hours, when all had disappeared.

Naturally the question arose as to whether the organism destroyed uric acid by means of a uricolytic enzyme or through more involved metabolic action. To test this point we secured a 24-hour growth of the

organism in broth. Then the culture was killed with chloroform and the organism separated in a centrifuge. The precipitate was ground thoroughly with powdered glass, then uric acid standard was added and the whole incubated with toluene for 24 hours at 37 C. A control of uric acid standard, ground glass, chloroform and toluene was similarly incubated. Since a slightly greater decrease in value was noted in the latter, we concluded that there was only the usual destruction of uric acid in an alkaline solution and that there was no evidence of the presence of an enzyme.

Confirmation of this conclusion was found in testing for uricase after the organism had grown in uric acid standard long enough (48 hours) to destroy all the uric acid. Chloroform was added and the organisms separated as before in a centrifuge. One c c. of the supernatant liquid was added to 4 c c. of the standard. After grinding the precipitate with powdered glass, it also was added to uric acid standard. In neither case did further destruction of uric acid occur.

TABLE 3

Destruction of Uric Acid by the Uric Acid Bacterium from Chicken Excreta. Medium-Phosphate Uric Acid Standard

Time of Growth	6 Hours	12 Hours	18 Hours	24 Hours	30 Hours	36 Hours	42 Hours	Control
Quantity remaining	100%	73%	55%	48%	44%	Trace	0	100%
Quantity destroyed	0	27%	45%	52%	56%	100%	100%	0

Since we employed a uric acid standard with and without acetic acid and not containing sulphate or chloride, it was to be expected that the majority of bacteria and particularly the pathogenic ones would fail to develop. Even in a more complicated synthetic medium as used by Koser, B. coli failed to grow. We tried to grow B. coli, B. paratyphosus, A and B, B. enteritidis, B. suipestifer, B. pullorum, Proteus vulgaris, B. aerogenes and Staphylococcus albus and aureus, but all these organisms refused to develop in the standard. In a future study we intend to adapt various groups of organisms to more complicated mediums containing uric acid as a source of nitrogen.

We added common molds and yeast to the series of bacteria studied and found that, though there was little growth in the standard, some destruction of uric acid took place. Several pathogenic molds were then planted on the standards. In table 4 appear those that were used on the phosphate-acetate standard, containing 1% glucose. Table 5 includes those planted on the phosphate standard to which had been

added 1% glucose and 1% glycerol. Under the former conditions Epidermophyton inguinale, Mastigocladium and nail Blastomyces destroyed only half of the uric acid in 14 days. Under the conditions of the second group Achorion violaceum, Sporotrichum gougeroti and an unknown skin mold destroyed 95, 30 and 100%, respectively, in 14

TABLE 4

DESTRUCTION OF URIC ACID BY PATHOGENIC MOLDS. MEDIUM: PHOSPHATE-ACETATE URIC ACID STANDARD, CONTAINING 1% GLUCOSE

Organism	6 Days			14 Days		
	Growth	Found by Analysis, Percentage	Destroyed, Percentage	Growth	Found by Analysis, Percentage	Destroyed, Percentage
Epidermophyton inguinale	Trace	100	0	Marked	50	50
Blastomyces-nail	Little	78	22	Marked	60	40
Mastigocladium blochi	Little	100	0	Marked	55	45
Trichophyton gypseum	Marked	100	0	Marked	100	0
Sporotrichum schenkii	None	100	0	.....	..	..

TABLE 5

DESTRUCTION OF URIC ACID BY PATHOGENIC MOLDS. MEDIUM: PHOSPHATE URIC ACID STANDARD, CONTAINING 1% GLUCOSE AND 1% GLYCEROL

Organism	14 Days			21 Days		
	Growth	Found by Analysis, Percentage	Destroyed, Percentage	Growth	Found by Analysis, Percentage	Destroyed, Percentage
Trichophyton gypseum I	Marked	107	0	Marked	104	0
Trichophyton gypseum II	Marked	101	0	Marked	96	4
Trichophyton rosae	None	104	0	None	108	0
Trichophyton cerebriforme	Little	100	0	Little	90	10
Achorion violaceum	Marked	5+	95—	Marked	0	100
Microsporon fulvum	Marked	102	0	Marked	100	0
Microsporon lanosum	None	98	2	None	96	4
Sporotrichum gougeroti	Marked	70	30	Marked	0	100
Coccidioides immitis	Little	102	0	Little	100	0
Epidermophyton inguinale	Little	100	0	Little	98	2
Skin mold, unknown	Marked	0	100	.....	..	..
Faviformis ochraceum	None	100	0	.....	..	..

days. The first two completed the destruction by the end of 21 days, while the 9 others studied refused to utilize more than negligible amounts by the end of the longer period. We intend to study other molds in order to add those that will utilize uric acid in the simplest standard solutions of uric acid here used and to determine other possible points of differentiation when other ions are present in the medium.

SUMMARY

Uric acid was destroyed in feces infusions under conditions that indicated activity of micro-organisms. Various bacteria were studied to determine their capacity for utilization of uric acid, usual uric acid standard solution being employed. Variations in phosphate and acetate used as solvents indicated that further modifications in medium would affect utilization. The uric acid bacterium of Ulpiani was isolated and quantitatively studied. Its power to destroy uric acid was not found to be due to uricase.

Several of a series of pathogenic molds definitely utilized uric acid though at a slower rate. Under the conditions of simple media Epidermophyton inguinale, Mastigocladium blochi, a nail Blastomyces, Achorion violaceum and Sporotrichum schenkii destroyed uric acid.

Further investigations with more complicated mediums are in progress.

BACTERIOLOGY OF URINE IN NEPHRITIS AND EXPERIMENTAL BACTERIAL NEPHRITIS *

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I. BACTERIOLOGY OF URINE IN NEPHRITIS

The reports of careful bacteriologic investigation of the urine in cases of nephritis are not numerous, and have dealt chiefly with acute rather than chronic conditions. In many instances there is an obvious infection preceding or accompanying an acute nephritis, while prolonged search in chronic cases may fail to reveal any hidden or obscure infectious element. Nevertheless, the impression prevails with many good clinicians that chronic nephritis is infectious in origin. With this in mind a careful search was made for evidence of infection in the urine of patients with nephritis who had been carefully examined for evidence of focal lesions.

In order to be of any value for bacteriologic study, the urine must be carefully obtained by catheter and culture made immediately. The presence of albumin in the urine, as Davis has shown, makes the urine an excellent bacterial medium favorable for the rapid multiplication of bacteria that may be present in the kidneys or bladder. The organisms found in cultures should also be demonstrable in the centrifuged sediment of the fresh urine. When urine is obtained and examined in this manner from normal persons it is found to be sterile in nearly all cases.[1]

The bacteria from urine most often reported in the literature have belonged to the streptococcus group. Thus Baehr [2] studied 34 cases postmortem and found 25 in which Streptococcus viridans was present. Since the streptococcus is an early postmortem invader such statistics should be correlated with antemortem findings before concluding that streptococci are etiologic agents in nephritis. Dick and Dick [3] reported 19 cases, carefully examined, in which streptococci were found 11 times, a diphtheroid one time, anaerobic bacilli—either feebly gram-positive or gram-negative—12 times, and a spore-bearing bacilli 2 times.

* Aided by a grant from the Fenger Memorial Association.
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[1] Jour. Infect. Dis., 1910, 7, p. 599.

[2] Jour. Exper. Med., 1912, 15, p. 330.

[3] Jour. Am. Med. Assn., 1915, 65, p. 6.

Campbell and Rhea[4] studied 13 cases of which 8 showed B. coli, one Staphylococcus aureus and 3 not stated. Ophüls[5] states that the etiology of acute and subacute nephritis is found to be due to bacterial infection and that this is quite probably true of the chronic form. The bacteria concerned are commonly but not necessarily members of the streptococcus family probably located in some hidden infection. No attempt will be made to review completely the literature on the subject.

This report covers 29 cases, 20 of which were chronic and 9 acute. In a large proportion of cases the urine was obtained by the writer by means of a sterile catheter. In all cases it was examined and plated within one hour. The examination consisted of a cell count, stain of the centrifuged sediment by Gram's method and methylene blue, the planting of 2 c c. of urine in 10 c c. of dextrose broth, 1 c c. in sheep blood agar plates, anaerobic culture on blood agar slants and shake cultures in dextrose agar. From these mediums subsequent stains were made and in many cases fermentative tests on Andrade carbohydate medium.

Reference to table 1 shows that in only 5 cases were cultures of streptococci obtained. In a surprising large number of cases (10), a feebly gram-positive organism was found, the strains of which fell fermentatively into 2 groups—one a typical nonfermenting diphtheroid, and one fermenting dextrose, lactose, saccharose, mannite and salicin but not changing maltose, insulin or dulcite. In 5 cases a spore-bearing organism was present. This was not a close relative of the subtilis group and was present in such numbers in the sediment of the centrifuged fresh urine as to exclude the probability of its being a contaminating organism. It did not peptonize gelatin or milk, grew readily in a large, moist "dew drop" colony, and fermented dextrose, lactose, mannite, maltose, inulin and salicin but not dulcite. Other organisms found were Staphylococcus aureus 6 times, an anaerobic Staphylococcus aureus 2 times, Staphylococcus albus once, B. coli 3 times and Diplococcus crassus once. Two specimens only were sterile.

It is realized that a history of focal infection can be elicited from a large number of adults with no evidence of kidney disease, so that a correlation of kidney disease with "hidden foci" cannot be definitely made in many cases. In a small percentage of cases definite improvement in the nephritis clinically occurs after the removal of a focus,

[4] Surg., Gynec. & Obst., 1918, 27, p. 611.
[5] The Pathology of Nephritis, 1916.

THE BACTERIAL FINDINGS IN THE URINE OF PATIENTS WITH NEPHRITIS

No.	Clinical Diagnosis	Cells per Cu. Mm. of Urine	Bacteria in Stained Centrifuged Sediment of Urine	Bacteria Found in Cultures	Remarks
1	Nephritis of pregnancy	7	Few slightly gram-positive bacilli	Slightly gram-positive bacilli	
2	Chronic parenchymatous nephritis	50	Few slightly gram-positive bacilli	Slightly gram-positive bacilli	Maxillary sinusitis; arterial hypertension; death
3	Chronic nephritis	7	Gram-positive cocci	Staph. aureus; slightly gram-positive bacilli	Identical results on 2 specimens 4 months apart; congenital syphilis; bronchitis
4	Acute hemorrhagic nephritis	430	Few gram-positive cocci	Staph. aureus; slightly gram-positive bacilli	Blood in urine persisted until after tonsillectomy 4 months later; Staph. septicemia; age 5 years
5	Chronic nephritis, uremia	600	Short bacilli	Short slightly gram-positive bacilli	Diabetes mellitus; puerperal nephritis; uremia; death
6	Chronic nephritis	15	Few slightly gram-positive bacilli	Slightly gram-positive bacilli	
7	Chronic nephritis	20	Few gram-negative bacilli	Streptococcus hemolyticus	Chronic tonsillitis; apical abscesses in teeth
8	Chronic nephritis	25	Gram-positive bacilli	Gram-positive spore-bearing bacilli	Syphilis; fibrinous pericarditis, terminal
9	Acute nephritis	140	Gram-positive bacilli	Slightly gram-positive bacilli	Age 13; measles 4 weeks previously; no atria of infection found
10	Acute nephritis	3,000	Gram-positive bacilli; few gram-positive cocci	Gram-positive spore bacilli; anaerobic staphylococci	
11	Chronic nephritis	20	Occasional gram-positive bacilli	Slightly gram-positive bacilli	Blood pressure 210 to 170 systolic; tonsils had been removed; no focal infection
12	Chronic nephritis	90	Gram-positive cocci	Gram-positive spore bacilli	Scarlet fever at 5 years; infected tonsils and antrum; tonsils removed; antrum drained; no improvement
13	Chronic nephritis	10	Gram-positive bacilli	Gram-positive bacilli with spores; few staphylococci	Blood pressure 165 to 185; teeth had been removed for 6 months; tonsils infected; 16 to 21% phthalein output
14	Acute hemorrhagic nephritis	50	Few gram-positive cocci	Staph. albus, anaerobic staph. aureus	Postscarlatinal
15	Chronic nephritis	10	Gram-positive cocci; slightly gram-positive bacilli	Slightly gram-positive bacilli; staphylococcus	Arterial hypertension; alveolar abscess; chronic seminal vesiculitis
16	Chronic parenchymatous nephritis	15	Slightly gram-positive bacilli	Slightly gram-positive bacilli	Tonsils infected and removed; no organisms like those in urine found in tonsils
17	Chronic nephritis	10	None	No growth	Tonsils infected and removed; many hemolytic streptococci in tonsils
18	Acute hemorrhagic nephritis	30	Occasional coccus	Streptococcus viridans	Clean specimen urine; age 14 years; followed cold and tonsillitis; tonsils removed and nephritis cured
19	Acute infectious nephritis	2,000	Many gram-positive cocci	Streptococcus viridans	Clean specimen urine; osteomyelitis 3 years previously; ethmoiditis and maxillary sinusitis; chronic otitis media
20	Acute nephritis	1,500	Gram-positive coccus	Staphylococcus aureus	Age 20; clean specimen urine; diphtheria 6 years before; tonsils infected; died
21	Acute nephritis	25	No bacteria	Anaerobic gram-positive bacilli	
22	Acute nephritis	350	No bacteria	B. coli	
23	Chronic nephritis	80	Many slightly gram-positive bacilli	Gram-positive spore bacilli	Repeated tonsillitis; tonsils out for 4 years; infection in nasal sinuses
24	Chronic nephritis	10	Occasional gram-negative	Diplococcus crassus	
25	Chronic nephritis	20	Gram-negative bacilli	B. coli	Trace of albumin; catheterized for 3 days previously; acute and chronic tonsillitis
26	Chronic nephritis	40	Gram-positive cocci	Streptococcus viridans	High blood chemistry; death
27	Chronic nephritis	...	.....	Streptococcus viridans	Age 27; infected tonsils had strep. viridans on removal; albumin diminished after tonsillectomy
28	Chronic nephritis	3,000	Gram-negative bacilli; few cocci ?	B. coli, Strep. viridans	Blood pressure 220; age 66; cardiac decompensation
29	Chronic nephritis	450	No bacteria	No growth	Death; Strep. viridans endocarditis

leaving little doubt of the relationship existing between the two conditions. It will be noted in table 1 that many of the 29 cases reported gave evidence of present or past foci of infection.

II. EXPERIMENTAL BACTERIAL NEPHRITIS

Experimental production of nephritis has been attempted by many workers by two methods: (1) the injection of known toxic chemical substances or of unknown toxic products; (2) the use of living or dead bacteria. Either method has met with success in producing acute kidney lesions comparable with acute nephritis in man, but neither method has met with uniformly positive results in producing chronic changes similar to chronic nephritis. One of the most difficult problems besetting the experimentor in chronic nephritis is to prove conclusively that lesions found at necropsy have been produced by the experimental procedures rather than by natural causes present in the experimental animal. Thus LeCount and Jackson [6] call attention to the fact that changes in normal rabbits were found similar to those present in animals injected with streptococci, although they found that many injected animals showed subacute changes essentially like those seen in acute interstitial nephritis of man.

It is a well-known fact that any of the salts of the heavy metals when injected in sublethal doses will produce acute changes in the tubular structure of the kidney; similar but less extensive changes are produced by less toxic chemicals. Underhill, Wells and Goldschmidt [7] and others have clearly shown that tartrate injections cause a nephritis effecting chiefly the convoluting tubules. Uranium nitrate has been found by many workers [8] to produce patchy, round cell infiltration and increased connective tissue growth in the rabbit. Arsenical preparations, potassium, dichromate, iodine, acid phosphate and other toxic agents have also been used experimentally to produce nephritis. These findings have suggested the possibility that chronic nephritis in man might be caused by bacterial infection in the kidney. To prove this experimentally, Faber [9] injected 200 M L D of diphtheria toxin into rabbits and found changes in the endothelial cells in the glomeruli. These changes were intensified by sublethal injections of B. coli or Vaughan's split protein from B. coli. Such changes were not noted by bacterial

[6] Jour. Infect. Dis., 1914, 15, p. 389.
[7] Jour. Exper. Med., 1913, 18, p. 322.
[8] O'Hare: Arch. Int. Med., 1913, 12, p. 49; Smith: Ibid., 1911, 8, p. 481.
[9] Jour. Exper. Med., 1917, 26, p. 139, 153, 707.

injection alone. Major [10] injected young rabbits with uranium nitrate and followed this by repeated injections of dead Staphylococcus aureus over a period of 4 to 6 months and found in 6 of 7 showed definite roughness and fibrosis of the kidney with round cell infiltration. Coulter and Pappenheimer [11] injected an extract from dead typhoid bacilli directly into the renal artery of animals sensitized previously to the extract and found glomerular changes after 48 hours. Streptococci and pneumococci as well as egg albumin were similarly used with positive results. Longcope [12] using egg albumin and horse serum in sensitized dogs and rabbits produced a nephritis with degeneration and necrosis of the epithelium of the loops of Henle, collecting tubules and occasionally in the convoluting tubules, followed by round cell infiltration and connective tissue overgrowth.

Bacterial injections alone have been repeatedly used in attempts to produce nephritis experimentally. Ophüls [13] is confident that bacterial foci of infection are responsible for nephritis and gives an excellent review of the earlier work on this phase of the subject. Since the streptococci from tonsillar infections have been frequently referred to in this relationship, Pappenheimer, Hyman and Zanan injected hemolytic streptococci into the renal vein and found acute glomerular lesions were produced. Winternitz and Zumby [14] injected bronchisepticus bacilli into the renal artery of dogs and found in young dogs a generalized infection followed by impairment of kidney function and renal changes similar to those of nephritis in man. Old dogs were apparently more resistant to the infection and showed fewer changes. Bloomfield,[15] on the other hand, found only slight evidence of renal changes when streptococci were injected into the renal artery of rabbits, and he feels that spontaneous nephritis is not excluded in many cases. Major [16] injected B. mucous capsulatus intravenously into rabbits and produced tubular necrosis, granular and hyaline casts with later fibrous changes when repeated doses were given. Rosenow and Meisser [17] have recently reported the production of nephritis by making an artificial focus of staphylococcal infection in the tooth of a dog.

[10] Jour. Med. Res., 1916-17, 35, p. 1349.
[11] Proc. N. Y. Path. Soc., 1916, 16, p. 80.
[12] Long Island Med. Jour., 1915, 9, p. 453.
[13] Jour. Am. Med. Assn., 1917, 69, p. 1223.
[14] Bull. Johns Hopkins Hosp., 1917, 28, p. 212.
[15] Ibid., 1919, 30, p. 121.
[16] Jour. Med. Res., 1917, 37, p. 125.
[17] Jour. Lab. and Clin. Med., 1922, 7, p. 707.

In my experiments, I have attempted to produce nephritis by repeated injections of living bacteria into rabbits and white rats. The bacteria used have been isolated either from the urine of cases of nephritis or from foci of infection, in the belief that such organisms might more readily induce a nephritis than stock cultures. The injections in rabbits have all been made intravenously once or twice weekly beginning with doses small enough so that rapidly fatal results would not follow. It was thought that by this means the likelihood of producing a chronic nephritis would be increased. The urine was frequently examined for albumin and casts. Usually sufficient urine could be obtained by massaging over the bladder, but occasionally catheterization had to be done. Following the first injection of living organisms, the bacteria could usually be recovered in the catheterized urine during the first 24 hours. After subsequent injections recovery was usually not possible. This points suggestively to an early destruction of the bacteria in the blood stream or other tissues as immunity is established and suggests most strongly the probability of bacterial toxins being directly concerned in any subsequent kidney changes.

The 16 rabbits used were each examined carefully before injection and were all found free from albuminuria. The bacteria used for injection were grown on blood agar slants and the growth washed off in normal salt solution. One twenty-fifth to $\frac{1}{5}$ of a slant was used as the initial dose and the dose increased gradually. When feebly growing streptococci were used, they were grown in dextrose broth and the broth suspension used for injection. Table 2 shows the experiment in detail. Four rabbits were treated with Streptococcus viridans, 4 with feebly gram-positive bacilli, 5 with Staphylococcus aureus, 2 with diphtheroid and 1 with B. coli. Eight of these were strains personally isolated from the urine of nephritis and described above, 6 from ethmoid infections, 1 from cystitis and one from sputum. Two of the rabbits survived only 1 injection, while 1 received 50 injections over a period of 14 months. Albumin in varying degrees was produced in all but one case, casts were present in 4 cases but are difficult to distinguish in the alkaline, heavily sedimented urine of rabbits.

The findings in the kidneys at necropsy were not striking. In every case there were slight variations from normal when sections of the kidney were examined microscopically. The usual changes in animals receiving 3 or more injections were destruction of tubular epithelium, infiltration and slight perivascular fibrosis. No marked secondary contraction of the kidneys were produced in any case.

TABLE 2

EXPERIMENTAL BACTERIAL NEPHRITIS IN RABBITS

Rabbit	Weight	Albumin	Casts	Total No. of Injections	Period Covered by Injections	Part of Slant Injected	Organism Injected	Source of Culture	Changes Found at Necropsy in Kidney
1	1,350	+++	Occasional granular	14	3 months	1/25 to 1/5	Streptococcus viridans	Sputum	Slight swelling of glomerular endothelium; destruction of cells of tubules; areas of round celled infiltration; slight increase in connective tissue cells; diagnosis: slight chronic interstitial nephritis
2	1,900 to 1,320	++	Negative	9	2 months	1/20 to 1/5	Diphtheroid chromogenic coccus	Chronic nephritis urine	No gross changes; glomeruli normal, convoluted tubules full of exudate, some epithelial cells destroyed, definite patchy increase in connective tissue; diagnosis: mild beginning chronic interstitial nephritis
3	1,600	Negative	Negative	1	1 day	1/10	B. coli	Cystitis	Endothelial cells in glomeruli swollen, exudate in convoluted tubules; diagnosis: acute glomerular nephritis
4	1,845	+++	Granular	18	2½ months	1/5 to 5	Slightly gram-positive bacilli	Chronic nephritis urine	Glomeruli normal; convoluted tubules full of exudate; intermediary zone shows increased connective tissue; diagnosis: chronic interstitial nephritis
5	1,410 to 950	+++	None	13	3 months	1/25 to 1/5	Staph. aureus	Ethmoid	Areas of round cell infiltration and increased fibrosis extending from cortex into medulla, shrunken glomeruli, fibrosis of tubular walls (patchy); diagnosis: diffuse chronic nephritis
6	1,340	+	None	3	1 week	1/20 to 1/12	Staph. aureus	Ethmoid	Glomerular hemorrhagic exudate; epithelium in tubules partly destroyed; small metastatic abscess; diagnosis: acute hemorrhagic nephritis
7	1,430	++	None	3	5 days	1/20 to 1/10	Staph. aureus	Ethmoid	Cloudy swelling; glomeruli swollen; many mycotic emboli tubules distorted and full of exudate; diagnosis: acute nephritis with emboli
8	1,850	++	None	1	2 days	1/25	Staph. aureus	Ethmoid	Marked swelling of kidney; glomeruli capsules full of exudate; endothelial cells swollen; interstitial hemorrhages; diagnosis: acute glomerular nephritis
9	1,750	++	None	50	14 months	1/20 to 6	Strep. viridans	Chronic nephritis urine	Grossly normal; areas of round cell infiltration; glomeruli shrunken; perivascular connective tissue increase; slight tubular changes; diagnosis: chronic nephritis
10	2,210	++	None	3	3 weeks	1/20 to 1/12	Diphtheroid	Ethmoid	Slight changes in convoluting tubules with epithelium denuded in places, slight round cell infiltration; diagnosis: slight chronic nephritis
11	1,120	+++	None	6	1 month	1/15 to 1	Slightly gram-positive bacilli	Chronic nephritis urine	Marked generalized round cell infiltration; increased connective tissue; tubules full of exudate; epithelium destroyed in places; hemorrhagic areas; diagnosis: marked chronic nephritis
12	1,420	++	None	5	1 month	1/5 to 1	Slightly gram-positive bacilli	Chronic nephritis urine	Increased connective tissues; round cell infiltration; perivascular connective tissue increase; some epithelium degeneration; diagnosis: definite chronic nephritis
13	1,490	+	None	13	3 months	1/20 to 1	Staph. aureus	Ethmoid	A few emboli; exudate into Bowman's capsule; connective tissue increase and hyperemia; tubules show areas of round cell infiltration; diagnosis; chronic nephritis with acute excerbation
14	1,650	++	Hyaline	20	3 months	1/5 to 5	Strep. viridans	Chronic nephritis urine	Increased connective tissue; round cell infiltration; slight tubular changes; diagnosis: chronic nephrits
15	1,660	+++	Few granular	25	4 months	1/25 to 6	Strep. viridans	Chronic nephritis urine	Mottled appearance; numerous emboli, glomeruli shrunken; connective tissue increase; diagnosis: chronic nephritis
16	1,420	++	None	3	3 weeks	1/10 to 1	Slightly gram-positive bacilli	Chronic nephritis urine	Small subcapsular hemorrhage; swollen cells in tubules; round cell infiltration in areas; hyperemia; diagnosis: acute glomerular nephritis

Since the normal reaction of rabbit urine is alkaline and can be made acid only with difficulty, it was believed that the use of a small omniverous animal like the rat might be more suitable for a study on experimental nephritis and would more nearly approach the conditions found in man. Preliminary studies were carried out to see whether the urine could be kept either alkaline or acid by dietary measure. It was found that rats on a bread, milk and vegetable diet almost invariable had an alkaline urine while on the same diet with meat added they had an acid urine. A straight vegetable diet was found to produce a constantly alkaline urine but also caused diarrhea, emaciation and death in 1 to 3 weeks. Albumin was found in a much higher percentage of animals whose urine was acid than in those with alkaline urine. Albuminuria was not constant from day to day in the same rat with a constant diet so that accurate figures cannot be given. The rats were divided into two groups: one group was fed an alkaline diet, and the other an acid diet. Each animal was injected subcutaneously twice a week with a non-pyogenic strain of staphylococcus which had an elective affinity for the kidney of rabbits. There was a distinct increase in the frequency of albuminuria in each group and frequently a change to acid urine in individuals of the group on an alkaline diet. The albuminuria in the acid group became more constant and judging by the urine alone a nephritis was being produced. At necropsy, however, no evidence of nephritis could be found. It seems probable that the higher protein diet of the acid group might be responsible for the albuminuria and that the bacterial injections played only a minor rôle.

SUMMARY

The urine in nephritic patients usually contains living bacteria.

The order of frequency of occurrence of these organisms, in the 29 cases reported, are: feebly gram-positive bacilli, Staphylococcus aureus, Streptococcus, spore-forming bacilli and B. coli.

Foci of infection can be found to be responsible for chronic nephritis in a very small number of cases.

Acute nephritis may be produced by the intravenous injection in rabbits of suitable doses of several varieties of bacteria.

Chronic nephritis in rabbits may be produced by repeated injections of bacteria but the changes in the kidney are never as striking as those seen in man.

Bacteria injected intravenously in rabbits are excreted in the urine following the first injections and may be readily cultivated. After repeated injections recovery is more difficult or impossible.

Albuminuria in rats can readily be produced by a high protein diet. This is not associated with marked changes in the kidneys.

Bacterial injection in rats increases the acidity of the urine and the frequency of albuminuria but did not cause changes in the kidneys in the cases studied.

TRYPANOSOMIASIS IN CAMELS

ITS DETECTION BY COMPLEMENT FIXATION

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In May, 1923, fifteen camels (single hump dromedaries) were offered for importation into the United States. The animals were placed in quarantine by the Bureau on arrival, and a sample of blood serum was obtained from each camel and forwarded to the Pathological Division, where the samples were tested by complement-fixation for trypanosomiasis.

Four of the 15 samples gave positive results, 2 gave indefinite reactions, and the remaining 9 gave negative reactions. The 6 (4 positive and 2 indefinite) camels were immediately placed in fly-proof quarters and the presence of trypanosomiasis was definitely established, the results of the complement-fixation test being confirmed by the isolation of the specific trypanosome from the camels by means of animal inoculations. The infected camels were then killed.

The remaining camels were held in quarantine and frequent fixation tests of their serums were made. The samples gave uniformly negative reactions.

Two rabbits were inoculated with the blood of each of these camels. Frequent blood examinations of the rabbits were made with negative results for trypanosomes, while 3 fixation tests of their serums resulted in negative reactions. These remaining camels, therefore, after a quarantine period of four months were considered free from infection, and were released.

In September, 1923, fifty-five camels were offered for importation from Australia. The serums of all of these camels gave clear-cut negative reactions to the fixation tests for trypanosomiasis. The negative reactions obtained with these serums act as a check on the specificity of the test as applied to camel serum.

Technic.—The same technic was employed as is used in the routine diagnosis of dourine.

The antigen[1] was prepared from Trypanosoma equiperdum, the causative agent of dourine, as it has been determined that fixation for trypanosomiasis is

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[1] Reynolds, F. H., and Schoening, H. W.: Jour. Agr. Res., 1918, 14, p. 573.

a group reaction. The surra trypanosome when used as an antigen fixes complement as well as the dourine trypanosome, in the presence of specific antibodies of dourine.

The serum was used in quantities of 0.1 and 0.2 c c., and was inactivated at 60 C. for 35 minutes in salt solution. Of the 15 camels imported, the serums of 4 (1, 12, 14 and 15) gave positive results; 2 (5 and 10) gave indefinite reactions, as the serum control tubes in 5 gave complete, and in 10, partial inhibition of hemolysis, as did the antigen tubes. The remaining samples (2, 3, 4, 6 to 8, 9, 11 and 13), gave clear-cut negative results to the test. The test was repeated the following day with identical results.

Isolation of the Trypanosome.—Blood was drawn from camels 1, 5, 10, 12, 14 and 15 into 0.5% solution of sodium citrate in salt solution and 5 c c. of this mixture from each of the 6 camels were injected subcutaneously into 2 rabbits. Daily blood examinations for several weeks failed to reveal the presence of the trypanosome in any of the rabbits.

Two rats were then inoculated with the blood of each rabbit, and trypanosomes were found in the blood of the rats which had been inoculated from rabbits 1 and 2 (camel 1), 7 and 8 (camel 5), and 9 (camel 14).

Two puppies inoculated with the blood of rabbits 7 and 8 (camel 5) and 3 inoculated with the pooled blood of all the rabbits became infected, and numerous trypanosomes were found in their blood. Trypanosomes were found later in the blood of the infected rabbits.

Grouping and Description of the Trypanosome.—The trypanosome isolated from the camels is believed to belong to the trypanosoma brucei group. This grouping was tentatively suggested by Sir Arnold Theiler on his visit to this laboratory from an examination of a stained preparation.

A few measurements of the trypanosome were made, but are of limited value, as they were made from only one preparation from the blood of a rat several days prior to death. The blood film was dried in air, fixed, and stained with Jenner's stain. The shortest of 40 trypanosomes measured was 26.4 microns and the longest 49.1 microns. The average length of the 40 was 37.3 microns.

An examination of a preparation of Trypanosoma equiperdum from a rat prior to death, fixed, stained and measured as the camel trypanosome, showed the shortest of 24 to be 24.1 microns and the longest 37 microns. The average length of the 24 measured was 30.3 microns.

The camel trypanosome is monomorphic, having a blepharoplast, nucleus and a well-marked flagellum. The blepharoplast is situated a short distance from the posterior end, which is markedly sharp or pointed. The nucleus in many of the stained specimens is situated slightly anteriorly, although in certain instances it is central. The free flagellum is decidedly long. The parasite is very active, but its translatory movements are restricted to the microscopic field. Undulating activity is quite marked.

Rat Pathogenicity Tests.—Rats inoculated with this trypanosome succumbed to infection in from 4 to 6 weeks.

A guinea-pig inoculated with blood of an infected rat on July 3, 1923, died on Nov. 2, 1923.

In the early stages, trypanosomes are found with difficulty in the blood of rabbits, although their presence can readily be demonstrated by inoculation of rats.

Dogs are susceptible to infection with this trypanosome.

On July 6, puppies 583 and 584 were inoculated with blood of rabbits 7 and 8, and trypanosomes were found in the blood on July 31.

On July 19, puppies 597, 598 and 599 were inoculated with the pooled blood of 12 rabbits. On Aug. 11, puppies 597 and 599 were dead. No examination of these animals had been made for trypanosomes prior to death. Postmortem examination of 599 showed all organs to be normal, while 597 had a spleen, from 6 to 7 times normal size.

Animals 583, 584 and 598 were alive on Dec. 26, 1923, and appeared to be in good condition. At times several of these animals showed a temporary posterior paralysis, which gradually disappeared.

Horse 237 was injected subcutaneously on Aug. 6, 1923, with the blood of dog 584, which on microscopic examination showed numerous trypanosomes. A marked rise in temperaure was noted from about every 5 to 7 days. The maximum temperature recorded was 106.8 F.

TABLE 1

RESULTS OF INOCULATION OF RABBITS

Rabbit	Date Inoculated	Results
1	June 12, 1923	Dead Dec. 13
2	June 12, 1923	Dead Dec. 17
3	May 23, 1923	Alive Dec. 17
4	May 23, 1923	Alive Dec. 17
7	June 12, 1923	Alive Dec. 17
8	June 12, 1923	Alive Dec. 17
9	June 12, 1923	Dead Dec. 28
10	July 3, 1923	Dead Sept. 6

Aug. 20, microscopic examination showed the presence of trypanosomes in the circulating blood.

The animal remained in good condition until Oct. 20, 1923, despite the high temperatures recorded at different times, when it developed a pronounced edematous swelling of the sheath and an edema along the abdominal wall. Examination of the edematous fluid of the sheath revealed the presence of trypanosomes. On Dec. 26, 1923, edema was still present, and the animal had fallen off some in flesh, but the appetite remained good.

On Sept. 5, 1923, cow 1027 was injected subcutaneously with the blood of dog 598, which showed numerous trypanosomes. The blood of the cow failed to show the trypanosome. That the animal was infected, however, was proved by the inoculation of rats and the demonstration of the trypanosome in the blood of these animals. The animal showed no physical evidence of infection. The temperature rose to 103.2 F. on Sept. 25 and 30, which were the highest points reached.

Sheep 607 was inoculated subcutaneously on Sept. 5, 1923, with the same material that cow 1027 received. Numerous microscopic examinations of the blood failed to reveal the presence of any trypanosomes. That the animal was infected, however, was proved by demonstrating the trypanosome in the blood of rats inoculated with the blood of the sheep. The temperature curve on this animal bore considerable similarity to that of cow 1027. The animal showed no physical evidence of trypanosomiasis.

Correlation Between the Complement-Fixation Test and the Isolation of the trypanosome from the Camels.—Two rabbits were inoculated with the blood of camels 1, 5, 10, 12, 14 and 15.

Serum of camel 1 gave a positive reaction to the test on 3 occasions, and both rabbits inoculated with the blood of this animal became infected with the trypanosome. This camel was killed.

Serum of camel 5 gave an atypical reaction on the 1st and 2d tests and a positive reaction on the 3rd test. Both rabbits inoculated with the blood of this animal developed the infection. The marked inhibitory properties of this serum in the first 2 tests evidently were a result of the presence of both antigen and antibody in the circulating blood resulting in a specific fixation of complement. At the time of the 3rd test the antigen evidently had disappeared from the blood stream, leaving only the specific antibodies. This camel was killed.

Serum of camel 10 showed a partial anticomplementary action on the 1st test. On the 2d and 3rd tests this anticomplementary action had disappeared,

TABLE 2

COMPLEMENT-FIXATION WITH HORSE AND COW SERUMS

Animal	Inoculated	Samples Drawn	Result of Test
Horse 237	Aug. 6	Aug. 4, 6, 9	Complete lysis
		Aug. 14, 17, 21, 24, 28, 31, Sept. 4, Nov. 27	Complete fixation
Cow 1027	Sept. 5	Sept. 5, 7	Complete lysis
		Sept. 11	Slight fixation
		Sept. 14, 19, 21, 25, Nov. 27	Complete fixation

resulting in negative reactions. Four rabbits inoculated at different times failed to develop the infection. This animal was released.

Serum of camel 12 gave a positive reaction on the 1st test and a negative reaction on the two succeeding tests. Both rabbits inoculated failed to develop the infection. In view of the positive reaction of the 1st test this animal was considered unsafe and was killed.

Serum of camel 14 gave a positive reaction to the 1st test and a negative reaction on the two succeeding tests. One of the two rabbits inoculated with the blood of this animal became infected. The other failed to develop the disease. This camel was killed.

Serum of camel 15 gave positive results to the test on three occasions, but both rabbits inoculated with the blood of this animal failed to become infected. This camel was killed.

Fixation Tests of Rabbit, Dog, Horse, Cow and Sheep Serums—Rabbit serum was inactivated at 62 C. for 35 minutes and used in quantities of 0.05 and 0.1 c c. Serum of rabbits 1 and 2 (camel 1), 3, 4, 7 and 8 (camel 5) and 9 (camel 14) gave positive results. The remaining serums gave negative reactions, together with the serums of a number of normal rabbits used as negative controls. Trypanosomes were demonstrated in all rabbits whose serums gave positive reactions.

The dog serum was inactivated at 60 C. for 35 minutes and used in quantities of 0.05 and 0.1 c c.

Serum of dogs 583, 584 and 598 gave a positive reaction to the test. The serum of 3 normal dogs gave negative reactions.

A sample of serum was obtained from horse 237, cow 1027 and sheep 607 prior to inoculation with the trypanosome. The animals were then inoculated, and samples were obtained at intervals of about 4 days. The serum was preserved with 0.5% phenol, and all samples were tested at the same time. The results of the horse and cow serums are given in table 2.

Horse 237 was inoculated on Aug. 6, and the serum gave a positive fixation test on Aug. 14. Trypanosomes were found in the blood on Aug. 20.

Cow 1027 was inoculated on Sept. 5, and the serum gave a positive reaction to the test September 14. Trypanosomes were not demonstrated in this animal by direct blood examination. The presence of the trypanosome, however, was proved by the inoculation of rats with the blood.

Specimens of serum of sheep 607 drawn on certain dates had a somewhat anticomplementary action. This was noted in the specimen drawn before inoculation, the anticomplementary action on certain of these drawings being apparently inherent in the serum and not a result of the inoculation.

The sheep gave a fluctuating antibody content, which for the most part remained comparatively low. On Oct. 2 a negative reaction was obtained, while on Nov. 27 the serum gave a positive reaction.

This may explain reactions obtained with serum of camels 12 and 14. On May 17 both of these samples gave positive results to the test, and on June 15 and July 10 both samples resulted negatively.

The serum of the rabbit, dog, horse and cow gave strong positive reactions to the test.

SUMMARY

The discovery of trypanosomias in these camels emphasizes the value of the complement-fixation test as a means of guarding against the introduction of trypanosome diseases into a country.

The use of the test in the diagnosis of dourine by the Bureau of Animal Industry during the last 10 years, during which time over 350,000 samples of serum from horses have been tested, has proved that the test is an extremely reliable diagnostic agent for this disease.

The results now recorded in which a trypanosome other than Trypanosoma equiperdum was involved, and serum from a species of animal other than the horse was used, indicate that the test is applicable as a diagnostic agent in ruminants affected with trypanosome of the T. brucei group.

While the fixation test for trypanosomiasis is a group reaction, it is believed from the results reported that the test should prove of value even in tropical countries, where a variety of trypanosomes exist, in assisting in the diagnosis of trypanosomiasis in man and animals. It would be of interest to determine the applicability of the test to the

blood of wild game as a possible means of readily determining the extent to which such animals act as reservoirs of infection.

The demonstration of the presence of specific trypanosomiasis antibodies may not necessarily indicate that the individual is harboring the parasite at the time of the test. An animal may recover, and it can be presumed that the antibodies elaborated may persist for some time in the blood stream after recovery.

ACTION OF LEUKOCYTES AND OF BRAIN TISSUE ON TOXIN OF B. BOTULINUS

WITH NOTE ON PEPTONE THERAPY IN EXPERIMENTAL BOTULISM

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The classical researches of Metchinkoff on the important rôle of the leukocytes in various infectious processes were followed by controversy for several years between him and his partisans and Buchner, Baumgarten and others opposed to his theories. This controversy was of immeasurable benefit for it gave an impetus to research which has elucidated many obscure problems of immunity and given results of vast benefit in the prevention and treatment of disease. Of late, interest in this subject has waned, especially as regards the direct action of leukocytes on soluble toxins. In the work of this character that has been done tetanus and diphtheria toxins have usually been employed.

Recent investigations of the many outbreaks of food poisoning due to B. botulinus have awakened a widespread interest in the powerful toxin of this organism. It therefore seems pertinent at this time to study any changes that may be produced in this toxin by the vital action of the leukocytes.

The early symptoms of the toxic action in man and animals of the products of B. botulinus parallel in some respects those of B. tetanus and a similar difficulty is encountered in treating botulism with specific antiserum, when definite symptoms have appeared.

I was lead to undertake this work by the unexpected results obtained while attempting to abort or ameliorate the symptoms of experimental botulism in guinea-pigs by the intramuscular injection of peptone (5%) in salt solution. Lüdke [1] appears to have had remarkable results in the treatment of 7 cases of tetanus, in which severe spasm had developed after incubation periods of from 7 to 11 days by injecting from 3 to 5 c c. of a 10% solution of deutero-albumose at from 24 to 48 hour intervals. All recovered in from 7 to 22 days. Kaznelson [2] also treated 2 cases of tetanus by injecting albumose, with one successful result. Table 1 shows that intramuscular injections (1.0 to 1.3 c c.) of 5%

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[1] Berl. Klin. Wchnschr., 1920, 57, p. 344.
[2] Ztschr. f. Klin. Med., 1916, 83, p. 275.

Witte peptone (albumose) at varying intervals subsequent to the subcutaneous injections of filtered botulinus toxin appeared to cause the death of the guinea-pigs in these experiments earlier than the controls.[3] Although the dose given may appear to be large, it is needless to state that the peptone injections alone were without appreciable effect on the control animals for they gained weight during the course of the injections.

In the study of nonspecific protein therapy against diphtheria toxin in guinea-pigs, Cowie and Greenthal[4] have shown a protective action

TABLE 1

INTRAMUSCULAR INJECTIONS OF 5% PEPTONE

Guinea-Pigs Weight	Toxin	Peptone	Symptoms	Result
471 gm.	.....	1 c c. 1⅓ hours 1 c c. 18 hours 1 c c. 43 hours 1 c c. 66½ hours	Gained weight	Survived
451 gm.	0.045 c c. fed.....	.....	No symptoms	Survived
441 gm.	0.045 c c. fed.....	1 c c. 1⅓ hours 1 c c. 18 hours 1 c c. 43 hours 1 c c. 66½ hours	Botulism Lost weight	Survived
340 gm.	0.1 c c. fed.....	.....	Botulism	Died 96 hours
345 gm.	0.1 c c. fed.....	1 c c. 3⅔ hours 1 c c. 21 hours 1 c c. 45 hours	Botulism	Died 51 hours
254 gm.	0.01 c c. subcutaneously.....	.....	Botulism	Died 55 hours
255 gm.	0.01 c c. subcutaneously.....	1 c c. ½ hour	Botulism	Died 15 hours
214 gm.	0.0066 c c. subcutaneously...	.....	Botulism	Died 16 days
187 gm.	0.0066 c c. subcutaneously...	1.3 c c. ½ hours 1.2 c c. 21 hours 1.3 c c. 43 hours 1.0 c c. 67 hours 1.0 c c. 90 hours	Botulism	Died 5 days
186 gm.	0.0033 c c. subcutaneously...	.....	Botulism	Died 11 days
195 gm.	0.0033 c c. subcutaneously...	1.3 c c. ½ hours 1.2 c c. 21 hours 1.3 c c. 43 hours 1.0 c c. 67 hours 1.0 c c. 90 hours	Botulism	Died 9½ days

of 3 c c. of normal horse serum against as high as 15 MLD of toxin. They later[5] showed this protection to be due to a natural antitoxin in the serum and not to the effect of the nonspecific protein injected. They also noted that 1 c c. of normal horse serum will neutralize one fatal dose of tetanus toxin. It was reported by Buxton[6] that he had

[3] Since this paper was written some experiments have been undertaken to influence the course of botulinus intoxication by various therapeutic measures. In guinea-pigs on an alkaline producing diet, the urine remained strongly alkaline throughout the course of the disease, except at necropsy when it was frequently found to be acid. The feeding or injections of small quantities of sodium bicarbonate or glucose appeared to hasten the fatal outcome, perhaps mainly on account of the frequent handling of the animals, although the controls which received salt solution or water and were handled to the same extent lived the longest.

[4] Jour. Med. Res., 1921, 42, p. 261.

[5] Ibid., 1922, 43, p. 21.

[6] Veterinary Rec., 1923, 3, p. 75.

demonstrated B. botulinus antitoxin in the blood serum of horses which had recovered from "grass sickness," but the serum of only one of 60 normal horses showed antitoxin (5 c c. neutralized 2 MLD). The death of some persons who have eaten food contaminated with botulinus toxin while the survival or freedom from illness of others who had presumably eaten the same amount of the contaminated food might reasonably lead us to suppose that aside from other factors, the serum of such persons may have contained a certain amount of antitoxin.[7] No protective effect was observed by Cowie and Greenthal[5] against diphtheria toxin by the serum of guinea-pigs nor with milk or egg white. All of these substances hastened the death of their animals; thus duplicating my results in the case of peptone injections. These authors conclude that nonspecific protein therapy is of value only in combating bacterial invaders and not in the destruction of toxin produced by them and they observe that when given late in the infectious diseases they do no good and may do harm. This is also my opinion so far as peptone therapy in botulism is concerned.

Just what takes place in the case of my attempted therapeutic peptone injections, I do not know. It seems more than likely that death was hastened by a prolonged negative phase during which the normal bodily defenses already so weakened by the toxin injections were further weakned by the slightly toxic peptone and the handling of the animals. Tromsdorf[8] has shown that a prolonged chilling of the body causes decreased mobility and phagocytic activity of the leukocytes. During the course of botulinus intoxication the temperature of the body usually remains subnormal and if the leukocytes do play a rôle in vivo in the neutralization of toxins as is so generally assumed, in the case of botulism their defensive action may be somewhat inhibited for this reason.

LEUKOCYTES, BRAIN TISSUE AND BOTULINUS TOXIN

Kobzarenko[9] in a detailed study of the effect in vitro of leukocytes on toxins obtained the following results: Fresh horse leukocytes possess the property of neutralizing diphtheria toxin in 4 hours, and this capacity does not depend on the physicochemical property of their

[7] It may be of interest in this connection to record my experiments with the serums of 12 men, 2 of whom were Wassermann positive. No protection was afforded by mixing 0.5 c c. of each serum with about 20 MLD of botulinus toxin and injecting subcutaneously into guinea-pigs although 3 animals receiving 1 negative and 2 syphilitic serums lived twice as long as the controls that received normal guinea-pig serum. I had previously found that 0.5 c c. of normal guinea-pig and rabbit serum failed to neutralize small doses of botulinus toxin.

[8] Arch. f. Hyg., 1906, 59, p. 1.

[9] Ann. de l'Inst. Pasteur, 1915, 29, p. 190.

protoplasm but on their vital activity. The same leukocytes are neither capable of absorbing nor neutralizing tetanus toxin. Only the leukocytes of rabbits containing from 15% to 20% of macrophages possess this property to a slight degree. Kobzarenko furthermore failed to neutralize diphtheria toxin with guinea-pig or rat leukocytes or with the macrophages of guinea-pigs after 1 hour's incubation. These results are in accord with those of Stenström.[10]

Mancini[11] found that tetanus toxin is not destroyed by horse leukocytes. These results were confirmed by Stenström,[10] who obtained negative results with the leukocytes of guinea-pigs and rats. Similar findings are recorded by Pettersson.[12] Wolff[13] concluded from his experiments that while guinea-pig leukocytes are capable of absorbing tetanus toxin, it is not neutralized by their action although Kobzarenk[7] was unable to confirm the results of these absorption experiments. Wordsworth and Vorhies[14] found that neither the leukocytes of dogs nor of guinea-pigs neutralize diphtheria or tetanus toxin. It would appear from this brief review that the leukocytes of the various animal species vary considerably in their action in vitro on the toxins of B. diphtheria and B. tetani.

It has been demonstrated by Hopkins and Parker[15] that blood cells show greater phagocytic activity for streptococci in serum than in salt solution, in which medium most in vitro experiments with leukocytes are conducted. On this account, and as the activities of the leukocytes after removal from the blood or exudates are probably greatly enfeebled, due to the peptone or aleuronat they may have absorbed, or the manipulations they have undergone, it is possible that a longer time would be required for neutralization to take place in vitro than in vivo, and an incubation of at least several hours should be allowed before any conclusive opinion could be given.

Technic

The toxin used in my experiments was obtained from a 4 or 5 days' growth of B. botulinus type A strain 89 in the beefheart medium of Dubovsky and Meyer[16] with the addition of liver to the medium. The cultures were filtered through a Mandler filter and the toxin kept in the icebox, usually in vacuo. The leukocytes for each experiment were obtained from the peritoneal cavity of guineapigs by the injection from 24 to 48 hours previously of a mixture of

[10] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1911, 8, p. 483.
[11] Biochem. Ztschr., 1910, 29, p. 140.
[12] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1911, 8, p. 498.
[13] Ibid., 1913, 18, p. 562.
[14] Jour. Immunol., 1921, 6, p. 413.
[15] Jour. Exper. Med., 1918, 27, p. 13.
[16] Jour. Infect. Dis., 1922, 31, p. 501.

alcuronat and peptone solutions. The peritoneal cavity was washed with warmed sodium citrate solution, the resulting leukocytic suspension centrifugalized and then washed 3 times in warm salt solution. After the final centrifugalization, the deposited leukocytes were measured and suspended in a known quantity of salt solution. The figures given in the tables for the amount of leukocytes used in the experiments represent the measured quantity of centrifugalized leukocytes. The toxin and suspension of leukocytes (1 c c. of each) were mixed, placed in the incubator at 36 C. for varying periods of time and the tubes shaken about every half hour. Toxicity tests of the toxin-leukocyte mixtures were made by subcutaneous injections into guinea-pigs. Toxin controls with 1 c c. of salt solution for each experiment were incubated the same length of time as the leukocyte-toxin-mixture, with frequently an extra control for the original toxin.

Experimental Data

Guinea-Pig Leukocytes.—The results of the action of guinea-pig leukocytes on filtered botulinus toxin was marked—almost without exception the potency of the toxin was greatly increased. The results appeared to be the same whether or not the leukocytes were centrifugalized out of the mixture before injection. A sojourn of several hours in the incubator of the toxin alone seems to diminish its toxicity (exper. 0 and 10).

The investigations of the various workers on the action of leukocytes on soluble toxins have shown that the toxicity is either unaffected, attenuated or destroyed. In none of the work reviewed has there been shown an increase in the toxicity. A determination of the chemical changes in the incubated leukocyte-toxin mixture would have to be made before any definite theory could be proposed as to the mode of action in vitro of guinea-pig leukocytes on botulinus toxin.

Action of Leukocytes in Vivo.—But little work seems to have been done to show whether an actual increase in the number of leukocytes at any given point in the body before the injection of toxins or the injection of leukocytes freshly mixed with toxin are of value in preventing or delaying intoxication. Kobzarenko[7] "prepared" rats for an influx of macrophages into the peritoneal cavity and found that they failed to neutralize diphtheria toxin subsequently injected. The results with macrophages in vitro were the same. As I had found that guinea-pig leukocytes markedly increase the toxicity of B. botulinus toxin in vitro, I thought it would be of interest to determine whether the same result could be obtained in vivo by the intraperitoneal injection of the toxin into guinea-pigs which had been "prepared" by the injection by the same route 48 hours previously of 5 c c. of 5% peptone (P_H 7.0). In order to obtain strictly comparable results a different stock of guinea-pigs reacting unformly has been used.

In the first series injected, 7 "prepared" and 7 controls, the animals all died about the same time, in from 22 to 30 hours. The "prepared" guinea-pigs survived a little longer than the controls. In the next series of 12 animals, all but one of the "prepared" animals survived the controls by about 15 hours, in spite of the fact that "prepared" animals are weakened by the peptone injection. The fact that these "prepared" animals survived longer than the controls was in all likelihood due to delayed absorption of the toxin caused by the inflammatory condition of the serous surfaces of the peritoneum or to the mucoid character of the exudate covering such surfaces. It is also

possible that the leukocytes themselves may have diminished somewhat the toxicity of the toxin.

Action of Brain Tissue.—In two experiments the brain tissue of a rabbit was finely ground in a mortar, salt solution added and 1 c c. of the suspension, equivalent to 0.4 c c. (approximately 0.3 gm.) of brain tissue, was mixed

TABLE 2

BOTULINUS TOXIN AND GUINEA-PIG LEUKOCYTES

Injections of 1 c c. Toxin and 1 c c. Leukocyte Suspension

No.	Weight	Toxin	Leukocytes	Hours at 36 Degrees	Injected	Symptoms	Survival
0	220	0.0005	0	0	Intraperitoneally	Botulism	36 hours
	230	0.0005	0	10	Intraperitoneally	Botulism	84 hours
1	383	0.00025	0.5 c c.	5	Intraperitoneally	Botulism	89 hours
	417	0.00025	.....	5	Intraperitoneally	Botulism	Survived
2	406	0.00025	0.1 c c.	5	Intraperitoneally	Botulism	48 hours
	410	0.00025	.....	5	Intraperitoneally	Botulism	Survived
3	429	0.0005	0.1 c c.	5½	Intraperitoneally	Botulism	17½ hours
	442	0.0005	.....	5½	Intraperitoneally	Botulism	89 hours
4	452	0.0001	0.1 c c.	6	Subcutaneously	Botulism	7 days
	465	0.0001	.....	6	Subcutaneously	Botulism	Survived
5	197	0.0033	0.1 c c.	4½	Subcutaneously	Botulism	36 hours
	156	0.0033	.....	4½	Subcutaneously	Botulism	11 days
6	214	0.0066	0.1 c c.	4¾	Subcutaneously	Botulism	3½ days
	214	0.0066	.....	4¾	Subcutaneously	Botulism	16 days
7	257	0.01	1.0 c c.	5	Subcutaneously	Botulism	24 hours
	254	0.01	.....	5	Subcutaneously	Botulism	55 hours
8	309	0.00036	0.05 c c.	5⅓	Subcutaneously	Botulism	17 hours
	253	0.00036	Sod. citrate*	5⅓	Subcutaneously	Botulism	68 hours
	274	0.00036	.....	5⅓	Subcutaneously	Botulism	46 hours
9	219	0.0009	0.6 c c.	11	Subcutaneously	Botulism	30 hours
	263	0.0009	.....	11	Subcutaneously	Botulism	Survived
10	270	0.00075	0.6 c c.	6	Subcutaneously	Botulism	42 hours
	250	0.00075	.....	6	Subcutaneously	Botulism	12 days
	235	0.00075	.....	0	Subcutaneously	Botulism	21 hours
	218	0.00075	.....	11	Subcutaneously	Botulism	Survived
	294	0.00075	0.6 c c.	11	Subcutaneously	Botulism	19 hours
11	330	0.0005	0.6 c c.	6	Subcutaneously	Botulism	58 hours
	306	0.0005	.....	6	Subcutaneously	Botulism	Survived
	309	0.0005	0.6 c c.	11	Subcutaneously	Botulism	45 hours
	301	0.0005	.....	11	Subcutaneously	Botulism	Survived
12	305	0.005	0.5 c c.	10⅓	Subcutaneously	Botulism	15 hours
	246	0.005	.....	10⅓	Subcutaneously	Botulism	46 hours
13	335	0.001	.....	....	Subcutaneously	Botulism	Survived
	341	0.001	.....	5	Subcutaneously	Botulism	Survived
	329	0.001	0.1 c c.	....	Subcutaneously	Botulism	4½ days
	320	0.001	0.1 c c.	5	Subcutaneously	Botulism	20 hours

* 1 c c. of sodium citrate solution. The increased protection afforded in this case is possibly due to the alkalinity of the citrate solution.

with 1 and 3 MLD of botulinus toxin. The mixture and controls were placed in the incubator at 36 C., shaken every half hour during 11½ hours, centrifugalized, and the supernatant fluid injected subcutaneously into guinea-pigs. There appeared to be no absorption of the toxin by the brain tissue. Using

mice in their toxicity tests, Kempner and Schepilewsky[17] found that 0.033 gm. of the brain tissue of guinea-pigs neutralized 1 MLD of botulinus toxin. They injected the entire brain toxin mixture but found that when centrifugalized the supernatant fluid was also nontoxic. Increased protection to the test animals was afforded when the mixture was placed in the ice chest for from 2 to 3 days.

I repeated the first mentioned experiment of these authors, carefully duplicating their technic except that I used the brain of rabbits. No neutralization of toxin occurred. I then repeated my own experiments using mice in my toxicity tests and incubating the toxin-brain mixture 17½ hours. No protection was afforded by this procedure even to those animals receiving less than 1 MLD, for they became just as ill at the same time as the controls.

Wordsworth and Vorhies[14] found that the brain tissue of guinea-pigs does not neutralize diphtheria toxin, although they were able to neutralize at least 5 MLD of tetanus toxin with about 0.05 gm. of this tissue. Wassermann and Takaki[18] have also shown the possibility of absorbing tetanus toxin with brain tissue. It is probable that this toxin has a special affinity for brain substance; as for other toxins, especially that of B. botulinus, the question of its neutralization may be somewhat dependent on the susceptibility of the animal species from which the brain for such tests was taken.

Coleman and Meyer[19] studying the pathogenicity of the spores of B. botulinus found that the brain emulsions of some of their intoxicated animals contained free toxin. It is possible, however, that this toxin was contained in the capillaries.

Semerak[20] states that botulinus toxin has no direct action on the nerve cells. Dickson and Shevky[21] show that the lesions in botulism are not of central distribution, but are peripheral and the blocking of nerve impulses must be due to some derangement which is relatively unstable and not to an organic break in the conduction apparatus. From a perusal of these papers, one would hardly expect to find that brain tissue would absorb botulinus toxin.

CONCLUSIONS

Intramuscular injections of peptone subsequent to the subcutaneous injection into guinea-pigs of botulinus toxin have no therapeutic effect and hasten the death of the animals.

Living leukocytes of the normal guinea-pig increase in vitro the toxicity of filtered cultures of B. botulinus. The action of botulinus toxin is delayed when injected into the peritoneal cavity of guinea-pigs in which an exudate rich in leukocytes has been produced by the injection of peptone.

The brain tissue of rabbits does not absorb botulinus toxin.

[17] Ztschr. f. Hyg. u. Infekhonskr., 1898, 37, p. 213.
[18] Berl. klin. Wchnschr., 1898, 35, p. 5.
[19] Jour. Infect. Dis., 1922, 31, p. 644.
[20] Ibid., 1921, 29, p. 190.
[21] Jour. Exper. Med., 1923, 38, p. 711.

RELATION OF REACTION OF INTESTINAL CONTENTS TO DIET AND FLORA

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There is some uncertainty as to the relationship between the diet and the H-ion concentration of the intestinal contents. General clinical and laboratory experience has shown that with an excess of fat or carbohydrate the reaction of the stools is acid, and with an excess of animal protein the reaction varies but tends to be alkaline. Recently, Rettger and Cheplin [1] have stated that when the flora has been entirely changed through the ingestion of lactose or acidophilus milk, there is still no increase in the acidity of the intestinal content, thereby controverting the claim that a simplified, gram-positive flora is coincident with an acid intestinal content. They, however, studied the reaction only of the feces and of a mixture of material from the cecum and the colon of experimental animals. They did not note separately the reaction of the intestinal content at cecal levels. Robinson [2] agreed substantially with Rettger and Cheplin that feeding lactose does not produce increased acidity apart from diarrhea, which introduces physiologic factors. Opposed to these views is that of Cannon and McNease,[3] who showed by feeding experiments with white rats that with the change in the fecal flora from a gas-producing, proteolytic type, there is a change in the acidity; and that the extent of the flora change toward the aciduric type varies directly with the increase in the concentration of the H-ion. They pointed out also that determination of the H-ion concentration of the feces is of little value in interpreting the reaction of the contents higher in the intestine. They believe further that the relation of the reaction in the cecum to the intestinal flora is of prime importance.

It was for the purpose of throwing more light on the relation of the intestinal reaction to the diet and flora that a repetition and an extension of some of the work mentioned were undertaken.

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[1] Intestinal Flora, 1921.
[2] Jour. Biol. Chem., 1922, 52, p. 445.
[3] Jour. Infect. Dis., 1923, 32, p. 175.

TECHNIC AND RESULTS

Healthy adult white rats were fed various diets for a week to 10 days. They were killed with ether, and their cecal and descending colonic contents were examined to determine the C-A ratio, the spirochete cecum-colon ratio, and the reaction. In a typical series presented here, 16 rats were fed in lots of 4 on four different diets for 10 days. During that period, all remained well and free from diarrhea. The 1st lot received lactose, milk, and bread; the 2d lot, a mixture of cooked ground beef, 3 parts, and lactose, 1 part, and water; the 3rd lot, the same meat and water; and the 4th lot, a stock diet of carrots, bread, oats, and water. To the meat diets was added a small amount of carrots. After the animals were killed, the contents of the cecum and lower colon were thoroughly and separately emulsified in sterile salt solution to an opacity matching tube 8 of the McFarland nephelometer scale.[4]

These emulsions were examined microscopically to determine the C-A ratio as a confirmation of the effect of the diet on the intestinal flora. It was done by making a large smear on a glass slide, staining by the standard Gram method, studying several typical fields, and carefully estimating the proportion of gram-negative bacilli of the B. coli group and the gram-positive bacilli of the aciduric group. These did not include all the bacilli of these staining reactions, but only those with the typical morphology of their respective groups. There was found a marked predominance of the B. coli group over the aciduric group in the protein and stock fed rats. Here also, the C-A ratio was usually higher in the descending colon than in the cecum, although there was not much difference in the acidity. The C-A ratio was low in both the cecum and the colon of the carbohydrate fed rats in which the concentration of the H-ion was appreciably greater than in similar regions of the other groups.

Other smears from the same emulsions were stained with Sterling's gentian violet and examined to obtain data concerning the presence of intestinal spirochetes and the cecum-colon spirochete ratio.[5] If it is true that their presence may be taken as an indication of putrefaction (Klarenbeek[6]), our finding no spirochetes in 3 rats and more in the colon than the cecum of the 4th carbohydrate fed rat indicates a

[4] Jour. Am. Med. Assn., 1907, 49, p. 1176.
[5] Parr: Jour. Infect. Dis., 1923, 33, p. 369.
[6] Abst. of Bacteriol., 1923, 7, p. 235 (Abst.).

fermentative and acid condition, as was borne out by the reaction found. On the other hand, spirochetes were found in the same sites in 9 of the 12 rats on high protein and stock diets, and in 6 of these the cecum-colon ratio was greater than 1. Apparently, the latter diets encourage the presence of intestinal spirochetes, especially in the cecum, which fact was coincident with reduced acidity in these regions.

After the smears were made for microscopic study, the emulsions were diluted further with distilled water for the purpose of determining the reaction. During the course of this work, we tried out the use of Mandler and Berkefeld filters in the endeavor to obtain quickly perfectly clear filtrates. The filters were new and carefully washed and rinsed in distilled water in which they were immersed for several days before use, the water being changed several times. The filters were assembled for suction filtration [7] and neutral water run through first. One-half of 10 samples were so treated. The other half of the same material, besides other samples, was cleared by adding a pledget of cotton and centrifuging at high speed for an hour. The H-ion of both preparations was determined by the use of suitable colorimetric indicators, comparing with standards freshly prepared from buffer mixtures recently checked electrometrically.

According to the tests of the centrifuged specimens, the reaction of the cecal contents of the rats on straight meat diet and on stock diet was nearly neutral, averaging P_H 6.8, while the colonic contents averaged P_H 6.6. When lactose was added to the meat, however, the cecal contents averaged P_H 6.4, but the colon material was less acid, P_H 6.9. This comparison was even more evident in the rats fed on a high carbohydrate diet, in which the cecal material averaged P_H 4.8, and the reaction of the feces was P_H 5.8.

The average concentration of the H-ion of 10 samples cleared by filtering was P_H 6.8, whereas the same samples cleared by centrifugalization gave an average of P_H 6.3. Most of this difference was found in samples from carbohydrate fed rats. The cecum of rat 87, for example, was P_H 4.8 by the centrifuge method and 6.8 by the filter method of clearing. In cases in which the emulsions to be tested were less acid, the two methods gave more comparable results. It thus seems that the weakly alkaline nature of these filters prevents their use when the material to be filtered is acid, if the preservation of that acidity is desired. It also suggests a factor in all experimental work on filtrable viruses and the sterilization of sugar solutions by filtration.

[7] Parr: Jour. Amer. Med. Assn., 1923, 80, p. 1775.

TABLE 1

THE RELATION OF THE REACTION OF THE INTESTINAL CONTENT TO THE DIET AND FLORA

Rat Number	Diet	The Reaction in Terms of P_H				C–A Ratio		Spirochaete Cecum–Colon Ratio
		Cecum		Colon				
		Filtered	Centrifuged	Filtered	Centrifuged	Cecum	Colon	
Group 1:	Lactose, milk, and bread							
85	.....	...	4.6	...	5.6	1:99	2:98	<1
86	.....	...	5.0	6.8	6.6	1:99	1:99	0
87	.....	6.8	4.8	...	5.5	1:99	1:99	0
88	.....	6.2	5.0	...	5.5	1:99	1:99	0
Group 2:	Meat, 3 parts; lactose, 1 part; water							
70	.....	...	6.5	...	7.0	25:75	80:20	0
71	.....	...	6.0	...	*	75:25	90:10	<1
72	.....	6.8	6.8	...	6.7	80:20	85:15	1
73	.....	7.2	6.4	...	7.0	85:15	95:5	>1
Group 3:	Meat and water							
75	.....	7.0	6.8	...	7.0	75:25	90:10	<1
76	.....	...	7.0	...	6.8	90:10	95:5	0
77	.....	...	7.0	...	6.8	*	*	>1
78	.....	6.8	6.9	...	6.8	75:25	90:10	>1
Group 4:	Carrots, bread, oats, and water (stock diet)							
80	.....	...	6.7	...	6.3	60:40	90:10	>1
81	.....	6.6	6.6	...	5.9	90:10	95:5	>1
82	.....	7.0	7.0	...	6.6	85:15	85:15	>1
83	.....	7.0	6.6	...	*	90:10	*	*

* Specimen missing.

SUMMARY AND CONCLUSIONS

A study was made of the effect of 4 diets on the reaction and the intestinal flora in groups of white rats. It was found that a diet containing sufficient carbohydrate to effect a complete turnover from a heterogeneous, gram-negative type of flora in rats fed on high protein and stock diets, to a flora markedly simplified and gram-positive, induced coincidently a change in the H-ion concentration from nearly neutral to distinctly acid.

In rats fed on a high carbohydrate diet, this acid environment was much more marked in the cecum than in the colon. In the same animals, however, even the contents of the descending colon were more acid than those of the rats fed on a high protein or stock diets.

Intestinal spirochetes were found in a larger number of rats on a high protein and stock diets, and in the cecum of these rats to a greater degree than in the descending colon, coincident with putrefaction and less acidity.

Filters in biologic operations should be used only with the understanding that they may alter the reaction of the material filtered, particularly if the original reaction be acid.

STUDY OF COMPLEMENT IN SERUM OF DIABETIC AND UREMIC PATIENTS

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The statement that diabetes predisposes to infection appears often in the literature, and the frequency of terminal infections in uremia suggests that a low resistance to infection also exists in this condition. With this in mind the present complement studies were undertaken.

Sweet,[1] after experimental pancreatectomy on two dogs, observed a loss of the hemolytic activity of the serum, and found in another series of experiments that this loss was due to the complement rather than to the amboceptor fraction. However, Sweet made his comparisons, not with the same dogs before and after operation, but with a diabetic dog and a normal animal, and, owing to the enormous individual variation in thermostable hemolysin among different animals, as noted by many immunologists, his data are inconclusive. There are, moreover, no standards by which to decide whether or not similar results might have been produced by other operations as extensive as pancreatectomy. Sweet asserts that complete pancreatectomy is necessary in order to secure such results, and this must be borne in mind in comparing his data with those obtained from patients with diabetes, in whom complete destruction of the pancreas is seldom, if ever, encountered. Glucose, per se, according to Handmann's [2] experimental observations, neither weakens the bactericidal power of the blood nor affects its opsonic index.

In view of the frequency with which acidosis complicates diabetes, the observations of Sherwood [3] on the effect of various acids on complement in rabbits are important. Sherwood compared the complement content and the hydrogen-ion concentration of the serum after anesthesia and following the injection of various acids, and obtained a drop in complement when the hydrogen-ion concentration was appreciably increased.

Urea causes a diminution in the activity of complement in vitro, according to Sherwood,[4] who found considerable variation in the

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[1] Jour. Med. Res., 1903-1904, 10, p. 255.
[2] Deutsch. Arch. f. klin. Med., 1911, 102, p. 1
[3] Jour. Infect. Dis., 1917, 20, p. 185.
[4] Ibid., 1922, 31, p. 252.

sensitiveness of complement from various animals, but learned that human complement was most easily inactivated, and that the effect often seemed to be permanent. From 0.02 to 0.04 c c. of a molecular solution of urea inactivated 1 unit of complement. Urea and complement were mixed, made up to 0.8 c c. with normal sodium chloride solution and incubated for one-half hour. Amboceptor and cells were added to make the volumes 1 c c., and the course of hemolysis was observed. If 0.02 c c. of urea were used during the first incubation, the concentration of urea to which the complement would be exposed would be 150 mg. for each 100 c c. of solution. If 0.04 c c. were used, the concentration of urea would be 300 mg. for each 100 c c. of solution. If complement is destroyed this would be the effective concentration of urea, but if it is only inhibited, the dilution with cells and amboceptor would increase the dilution of the urea so that the concentration would equal only 120 or 240 mg. for each 100 c c. of solution, depending on the amount of urea used. Such concentrations are well within the range of those cases of uremia in which there is marked nitrogen retention. Sherwood also injected rabbits intravenously with from 2 to 6 c c. of a molecular solution of urea, and found that when the blood urea rose from 47 to 60 mg. the complement dropped. Such acute experiments are, however, not comparable to the chronic conditions of uremia which occur clinically, and which allow time for adjustment of the organism itself to the continued presence of excessive nitrogen fractions. Sherwood does not claim that these results apply to the cases of uremia observed clinically. Beyond these few reports, so far as we have been able to determine, the literature contains nothing that bears directly on this subject.

METHOD OF STUDY

Reagents were prepared freshly and titrated daily in accordance with the Kolmer [5] technic for the Wassermann test. The blood was drawn from the patient and placed on ice immediately. Most of the tests were made 7 or 8 hours later; all were made within 24 hours. In the first titration, 0.5 c c. of serum was placed in the first of 8 tubes, with 0.5 c c. of sodium chloride solution. These were mixed, and 0.5 c c. of the mixture was pipetted to the next tube, and so on, until the last tube was reached, when 0.5 c c. was discarded. This gives a total final dilution of patient's serum in each tube as follows: 1:6, 1:12,

[5] Am. Jour. Syphilis, 1922, 6, p. 82.

1:24, 1:48, 1:96, 1:192, 1:384 and 1:768. Two controls were used with each test, one of the cells with amboceptor and the other with complement. Like many other observers, we found a marked variation in the amount of natural thermostable amboceptor present. This resulted in a variation from no hemolysis to complete hemolysis in the control tube. Because of the constancy of our results, however, we believe this factor to be negligible. All tests were made in duplicate and most of them checked exactly. The difference was never greater than that of partial hemolysis from one tube to the next, hence only one reading is recorded here. The first reading was taken after incubation for one hour in the water bath. The racks were then put in the icebox over night and read again after the cells had settled, the only difference being that the relative amount of cells and hemoglobin was made clearer in some of the tubes in which hemolysis was incomplete. After the complement had been determined in the serum of 15 patients by this method of titration, another method was substituted for the remainder of the work. By this method 10 tubes were used containing from 0.2 to 0.01 c.c. of complement, and finer variations in the strength of the complement could be determined. The blood sugar was determined by the Folin-Wu method, and the carbon dioxide capacity by the Van Slyke apparatus on each of the specimens of serum examined. The total blood fat of the whole blood and plasma was determined by the method of Bloor, modified according to the suggestion of Denis, so that the readings are made in a colorimeter instead of in a nephelometer, as described by Bloor.

LIPEMIA AND THE DETERMINATION OF COMPLEMENT

The most marked case of lipemia is illustrated in case 1 (table 1). Because of the high fat content (7.67%) in the tubes, it was difficult to read them, but at first sight, apparently there was no hemolysis in

TABLE 1

LIPEMIA

Case	Date	Blood Sugar, Mg.	Carbon Dioxid, Percentage	Total Fat*	1	2	3	4	5	6	7	8	9	10
1	4/28	157	59	7.67 (P)	H	H	H	H	H	H	H	H0	0H	0
	4/30	400	32	.....	H	H	H	H	H	H	H	H0	0H	0
2	....	257	38	3.54 (P)	H	H	H	H	H	H	H	H0	0H	0
3	....	350	30	3.90 (B)	H	H	H	H	H	H	H0	H0	0H	0
4	....	213	26	2.0 (B)	H	H	H	H	H	H	H	H0	0H	0

H means complete hemolysis, 0 no hemolysis, and 0H and H0 partial hemolysis, the initial letter showing which predominates.

* Total fat in whole blood (B), or plasma (P).

the first 5 or 6 tubes. However, hemolysis appeared in all the tubes on simple dilution of the fluid with isotonic sodium chloride solution. Microscopic examination of the contents of the first tube in the rack in which hemolysis should have been complete, if in any, revealed erythrocytes, but few in comparison with those originally present. Evidently, then, while there may have been a slight inhibition by the action of the fat particles, hemolysis had, in fact, occurred, but was obscured by the fat emulsion. In case 2 the findings were practically the same; a few erythrocytes were seen under the microscope. In case 3 hemolysis could not at first be demonstrated in any of the tubes, but it appeared in all on dilution. However, no erythrocytes could be seen under the microscope, so hemolysis was apparently only obscured. In all of these cases the end points were the same as in the others, both before and after dilution. The same trouble, due to the lipemia, occurred in reading the result of the Wassermann test, and the result was made visible by simple dilution. If the reactions had been read as usual without this additional study, they would have been reported as anticomplementary. This may possibly be the explanation of some of the anticomplimentary Wassermann reactions reported in cases of diabetes. It will be noted that the amount of apparent inhibition which occurs before dilution is not proportional to the amount of fat present. In case 3, in which none of the tubes could be read, there was only about one-half as much fat as in case 1 in which the apparent inhibition was much less. Apparently the state of emulsion of the fat is an important factor. We have noticed the same fact in comparing the amount of blood fat necessary to produce visible lipemia retinalis.

Koenigsfeld [6] has reported a drop in complement, paralleling the rise in cholesterol in lipemia. However, he worked only with experimental lipemia induced in animals by repeated bleeding. This is not at all comparable to the more chronic form of lipemia seen in patients with diabetes. Until we know more about the fluctuations of complement after hemorrhage and without lipemia, no definite conclusions can be drawn.

RESULTS

Since our results are experimentally negative, we have condensed our comments on them to the following brief statements:

The complement content in the serum of diabetic patients is remarkably constant in spite of severe metabolic disturbances. Thirty

[6] Ztschr. f. d. ges. exper. Med., 1922, 29, p. 190.

patients were tested, 15 by each of the two methods described. The blood sugar in these cases varied from 0.85 mg. to 0.345 mg. for each 100 c c. of blood, and the carbon dioxide capacity from normal to as low as 31%. The complement content showed no significant variation. These results were controlled by determinations of 10 normal persons.

TABLE 2

SEVERE ACIDOSIS

Case	Blood Sugar, Mg.	Carbon Dioxid, Percentage	1	2	3	4	5	6	7	8	9	10
1	307	14	H	H	H	H	H	H	H	H0	0H	0
2	220	15	H	H	H	H	H	H	H	H	H	0H
3	400	17	H	H	H	H	H	H	H	H0	0H	0H
4	...	24	H	H	H	H	H	H	H	H	H0	0
5	455	24	H	H	H	H	H	H	H	H0	0H	0
6	...	24	H	H	H	H	H	H	H	H0	H0	0H
7	339	27	H	H	H	H	H	H	H	H	H0	0H

Seven patients with severe acidosis, some with complicating infections, were observed (table 2). Dehydration was a marked clinical feature. There is an apparent slight increase in complement in some of these cases, which may be explained by the concentration of the colloids from loss of water.

TABLE 3

INFECTIONS

Case	Diagnosis	Blood Sugar, Mg.	Carbon Dioxid, Percentage	1	2	3	4	5	6	7	8	9	10
1	Acute osteomyelitis and arthritis of toes	317	56	H	H	H	H	H	H	H	H0	0H	0
2	Purulent epididymitis	328	58	H	H	H	H	H	H	H	H	H0	0
3	Perforating ulcer of foot	282	48	H	H	H	H	H	H	H	H0	H0	0
4	Diabetic gangrene	317	51	H	H	H	H	H	H	H	H0	H0	0
5	Mild furunculosis and impetigo; duodenal ulcer	371	39	H	H	H	H	H	H	H	H	0H	0
6	Diabetic gangrene	141	63	H	H	H	H	H	H	H	H0	0H	0
7	Staphylococcus septicopyemia	275	46	H	H	H	H	H0	H0	0H	0	0	0

The results with 7 other diabetic patients with infection uncomplicated by acidosis are shown in table 3. In only one case (case 7) is there an alteration in complement. This patient had severe staphylococcus septicopyemia, and this was probably responsible, rather than the diabetes and acidosis, for the fall in complement.

The results in cases of lipemia have been discussed.

The Wassermann reaction by the Kolmer technic was negative in all of these cases.

The complement in 9 cases of uremia with marked nitrogen retention was without significant abnormality (table 4).

TABLE 4

Uremia

Case	Urea, Mg.	Uric Acid, Mg.	Creatinin, Mg.	Complement Titration, Tube Numbers, Serum, Percentage, C c.									
				1 0.2	2 0.17	3 0.15	4 0.13	5 0.10	6 0.07	7 0.05	8 0.03	9 0.02	10 0.01
1	102	7.6	5.9	H	H	H	H	H	H	H	H0	0H	0
2	107	4.2	3.1	H	H	H	H	H	H	H	H0	0H	0
3	134	5.0	6.1	H	H	H	H	H	H	H	H0	0	0
4	163	7.0	7 0	H	H	H	H	H	H	H	H0	0H	0
5	168	5.9	11.4	H	H	H	H	H	H	H	H	0H	0
6	219	4.65	11.3	H	H	H	H	H	H	H	H0	0H	0
7	239	6.7	9.3	H	H	H	H	H	H	H	H0	0	0
8	312	8.3	15.0	H	H	H	H	H	H	H	H0	0H	0
9	383	10.6	13.6	H	H	H	H	H	H	H	H0	0H	0

SUMMARY

Forty-eight patients with diabetes and 9 with severe uremia showed no significant variation in the complement content of their serum.

PRODUCTION OF MYCELIAL FORMS BY OÏDIUM ALBICANS IN CARROT INFUSION

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Oïdium albicans (Monilia albicans) is considered the specific cause of thrush or parasite stomatitis.

The actual classification of the organism has been a subject for discussion since it was first isolated by Robin.[1] The formation of long branching mycelial forms similar to the morphology of the molds on one medium and the formation of yeasts on other mediums has been the ground for dispute. Although it has had numerous names given to it, the organism is more generally classed as an oïdium.

When found in the false membrane of thrush, generally the mycelial forms predominate. The cells are elongated and at the junction of these, other cells bud forth and in turn take on the mycelial growth. Transplants from the membrane to ordinary mediums yield the yeast forms.

The purpose of this paper is to report the methods used in an attempt to discover the cultural conditions necessary for the production of the mycelial forms.

Guilliermond [2] states that cultures on carrot develop quite a mycelial structure. Grawitz [3] believed that the mycelial forms of the organism were only a form that it acquired when placed under conditions that were unsatisfactory, inasmuch as on bringing the form back to its proper nutritive medium, the mycelia will return to the true budding variety.

Roux and Linnossier [3] supported Grawitz by reporting that the "forme mycelienne" existed only under unfavorable conditions.

On the other hand, when considering this organism in its relation to thrush, it is apparent that the mycelial forms are present under favorable conditions, and on being cultured on artificial mediums, they transform themselves into yeasts.

Roux and Linnossier grew the organism on melon with abundant mycelial formation, although, from their description of the cells, they

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[1] Histoire naturell des vegetaux parasites qui croissant sur l'homme et sur les animaux vivants, 1853, p. 488.

[2] Yeasts, translated by Tanner, 1920, p. 361.

[3] Arch. de méd. éxpér., 1890, 2, p. 64.

were considerably shorter (3-5 microns in length) than those generally isolated from thrush. They also report cultivation of the organism on stewed carrot, but they did not observe any formation other than the yeast structures. Guilliermond suggests sliced raw carrot as a medium for mycelial formation. However, I noted only rudimentary filaments on this medium.

PERSONAL OBSERVATIONS

A culture of Oïdium albicans was isolated from the stool of a patient with chronic diarrhea. In the stools, the mycelial forms pre-

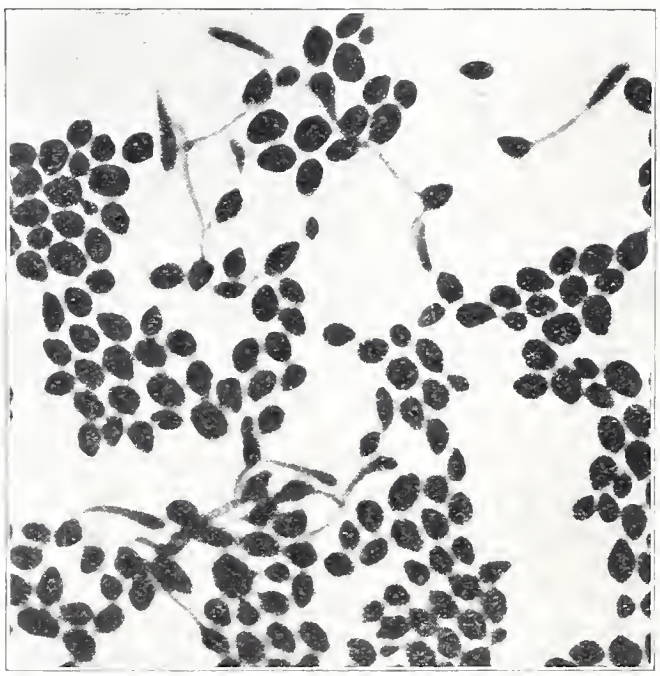

Fig. 1.—Rudimentary filaments in a 24-hour culture on solid carrot. × 1,000

dominated, often occurring as tangled masses of hyphae. As usual, the first subcultures yielded the yeast forms.

Assuming that a slight acid reaction of the medium might bring about some morphologic variation, several organic acids were added, separately, to plain meat infusion agar (P_H 6.5). The following proportions of acids were added to the medium: To each 10 c c. of melted nutrient agar there was added 0.05 c c. of saturated solutions of malic, malonic, succinic, lactic and oxalic acids, and the plates poured. They were incubated for 20 days at 35 C. Microscopic examinations were

made daily on and after the 8th day. The growth was abundant, and from cultural appearances the medium proved favorable. However, morphologically, the oïdium grew in only the yeastlike forms.

Following the same technic, the percentages of the acids were increased from 0.5 to 2 at increments of 0.05% each. The lactic acid cultures of acidity of from 0.5 to 0.75%, grown for about 9 days, showed small rudimentary filaments. These ranged from 6 microns to 8 microns in length and were as wide as the mother cell itself, ranging from 1.5 microns to 2.5 microns. Seldom were more than one or two filaments joined together.

A similar experiment was conducted using N/20 Na OH in place of the acids, on plates containing 0.4 c c.-0.8 c c. of the alkali and those

TABLE 1

RESULTS OBTAINED WITH METHOD OF ROCKWELL

Parts O_2	Parts CO_2	Growth
1	2	Fair
2	3	Fair
3	5	Fair
1	1	Good
3	2	Good
2	1	Abundant
5	3	Abundant
1	9	Abundant
"	1	None

containing 1.7 c c. to 2 c c. more developed hyphae were observed. It was interesting to note that the mycelial forms did not appear on cultures containing 0.8 c c. to 1.7 c c. alkali.

The organism was then grown on the various common mediums, such as agar, gelatin, blood serum, potato, carrot and sugar agar. All cultures were grown under partial oxygen tension without showing mycelial forms.

The following table will illustrate the results obtained by growing the organism under various mixtures of O_2 and CO_2 according to the method used by Rockwell.[4]

Microscopic examinations of the organism grown under these conditions failed to show the presence of mycelial forms.

The organism grown on carrot (approximately P_H 7) produced the filaments noted by Guilliermond. However, the chains were limited to from 2 to 4 cells. The filaments appeared to be rudimentary and about to disintegrate (fig. 1). The best results were obtained by grow-

[4] Jour. Infect. Dis., 1923, 32, p. 98.

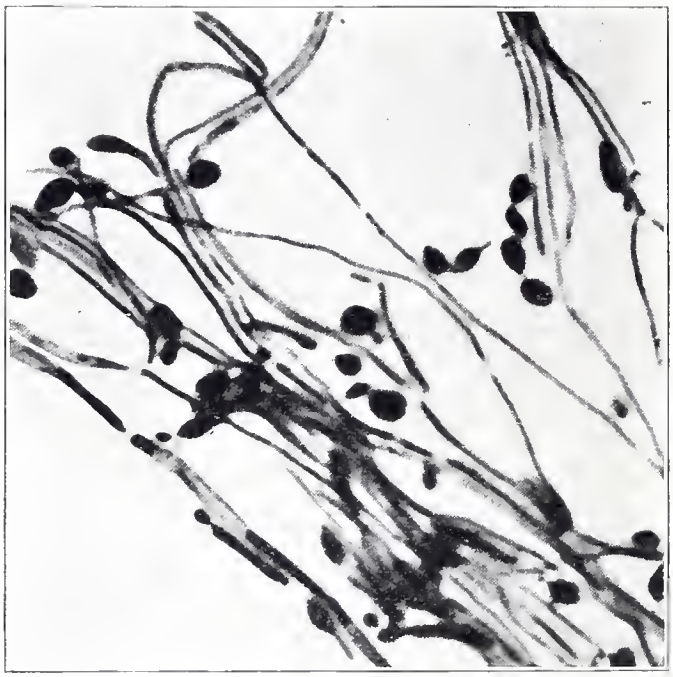

Fig. 2.—Well developed branching filaments in a 48-hour culture in carrot infusion. × 1,000.

Fig. 3.—Like fig. 2, but showing in detail the method of bud and branch formation. × 1,000.

ing the organism on a carrot infusion. This medium was prepared by chopping raw carrots into fine particles and allowing to extract in sterile distilled water at about 10 C. for 48 hours.

The extract was then filtered, tubed and autoclaved for 15 minutes at 15 pounds pressure.

The oïdium introduced into this medium produced a good growth in 12 hours. The first noticeable growth appears to adhere to the side of the test tube, but on slight agitation drops to the bottom and continues to develop with a "downy" appearance. Microscopic examination reveals long chains of filaments after 24 hours of incubation at 37.5 C. The cells averaged in length from 6 microns to 14 microns. In young cultures, practically all the cells were changed from the yeast cells to mycelial forms (figs. 2 and 3).

Smears made from these cultures resembled in every way those made from the original stools. The morphology is most easily studied either in fresh preparations or by fixing the smear while wet with osmic acid and staining with dilute carbol-fuchsin or with Archibald's stain.

SUMMARY

An attempt was made to find conditions favorable for the formation of mycelial in cultures of Oïdium albicans. A few rudimentary filaments were formed in certain concentrations of acid and alkali on an agar medium. Only rudimentary filaments also were found on the solid carrot medium recommended by Guilliermond.

In an infusion of carrot, however, typical hyphae developed, and these closely resembled the forms found in the lesions of thrush.

COLLOIDAL INHIBITION OF ANAPHYLACTIC SHOCK

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A previous study [1] dealt with the inhibition of anaphylactic shock by the intravenous injection of heterologous serums shortly before the toxic dose of homologous or sensitizing serum. Interpretations based on the method employed indicated that a refractory state was induced. At that time we referred to the refractory state as desensitization, but since we were not dealing with pure proteins and since the refractory condition was not specific, we now employ the term inhibition. Our work is directed ultimately toward the problem as it affects man, and for this reason we have studied sensitization with blood serum rather than with pure proteins. The method employed in the previous study has been used in a large part of the present study. Sensitized animals were given intravenous injections of homologous serum in order to determine a small lethal dose. In common with many other observers, we regarded a dose as almost uniformly fatal if it killed a series of 3 of 4 guinea-pigs. Accordingly, if the injection of an agent shortly before the toxic dose of serum was followed by recovery of a large proportion of the animals, it was assumed that inhibition had been effected. This will be referred to again in the discussion of our results.

Many investigators have published reports which indicate that in anaphylactic shock there is a disturbance of colloidal balance of the body, responsible for the shock. If this be true, it is reasonable to assume that specific desensitization is accomplished by survival of such a colloidal disturbance, quantitatively insufficient to produce death. The present study is an attempt to determine whether or not colloidal disturbances produced in other ways might serve also as inhibitory without exposing the animal or the patient to the dangers or loss of time incident to specific desensitization. Admittedly similar disturbances may be produced by colloids, crystalloids and alterations of hydrogen-ion concentration. Hanzlik and Karsner [2] have shown that the intravenous injection of a wide variety of colloids may produce symp-

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[1] Karsner, H. T., and Ecker, E. E.: Jour. Infect. Dis., 1922, 30, p. 333.

[2] Jour. Pharm. & Exper. Therap., 1920, 14, pp. 379, 425, 449, 479; Proc. Soc. Exper. Biol. & Med., 1922, 19, p. 302.

toms resembling those of anaphylactic shock, but with different physiologic and anatomic findings. Familiarity with these substances determined us in selecting the use of colloids in this phase of the study. We have therefore employed Congo red, dextrin, Witte pepton, glycogen and kephalin, which were found by Hanzlik and Karsner to be nonfatal. The experiments will be reported in two groups.

The technic was uniform in both groups. Animals were sensitized by subcutaneous injection of 1 c c. 1 : 20 dilution of horse serum, the small lump so produced being carefully massaged. The subsequent experiments were made in from 20 to 25 days after sensitization. The injections of colloids were made by isolating the jugular vein, tying the needle in the vein, injecting by a syringe, subsequently ligating the vein and closing the skin with a Michel clip. The injections of horse serum were made in the same manner on the opposite side. Freezing the skin with ethyl chloride rendered the operation painless. The animals were observed in small separate cages and the necropsies performed within a few minutes after death. In order to assure maximum speed of work, this required the attention of both authors, two technicians and our colleague, Dr. F. P. Lyne.

SERIES A

Congo Red.—This is a suspensoid colloid, which in doses of 0.065 mg. per gram animal weight was fatal in our earlier work. Death appeared to be respiratory rather than circulatory. In the present series, doses of approximately 0.005 and 0.01 mg. per gram animal weight were employed and were fatal for 3 of 13 animals. Seven of the remaining animals were given 0.2 c.c. each of homologous serum (horse) one hour after the Congo red (1% and 0.5% suspension), and 6 died promptly of anaphylactic shock with typical necropsy findings. Three were given the toxic dose of horse serum 24 hours after the Congo red, and 2 of these died in typical shock with characteristic necropsy findings. In the control series, 0.1 cc. of horse serum had been fatal. Even with the most liberal interpretation of individual variations in animals, the weights being 485 gm. ± 25 gm., it is probable that Congo red exercises no inhibitory action on anaphylactic shock.

Dextrin.—This is a nonprotein colloid, which was not fatal in doses as high as 1 mg. per gram of animal weight in the earlier studies, but produced slight distention of lungs and pronounced circulatory disturbance. We employed in this series doses of 0.4 and 0.8 mg. per gram animal weight, about the same doses as in the earlier series. These were not fatal and produced only slight reactions in 8 of 12 animals. Two of these died, however, during the following night. Of 6 animals given the toxic dose, fatal for 2 control animals, only 2 died and exhibited typical symptoms and necropsy findings. The 4 surviving animals showed in 3 instances moderate shock and in the other severe shock. Four animals survived the injection of dextrin 24 hours, and all of these survived the toxic dose of homologous serum. The animals weighed 350 gm. ± 50 gm., and the 2 controls, weighing respectively 400 and 318 gm., died of the

toxic dose (0.2 c c. horse serum) with typical shock and necropsy findings. Assuming that the animals were equally sensitive, there is moderate protection by the use of dextrin. As will be pointed out subsequently, this assumption is not justified.

Glycogen.—This is a colloid, which in doses of 0.05 and 0.065 mg. per gram animal weight was not fatal in the earlier studies, but produced severe respiratory distress and showed at necropsy pulmonary distention, although perfusion failed to show bronchial constriction. The effects are on both respiratory and circulatory systems. The doses in this series were 0.025 and 0.05 mg. per gram animal weight, which were followed by slight or moderate reactions on the part of 10 of the 14 animals employed. They were treated 22 days after subcutaneous sensitization with horse serum. Seven animals were given the toxic dose of serum 45 minutes after injections of glycogen, 3 following the smaller doses of glycogen and 4 following the larger doses. Of the 3 with smaller doses, all died typically. Of the 4 with larger doses, 3 died typically. Twenty-four hours after the glycogen, 3 given smaller doses were killed typically by the toxic dose of horse serum, whereas of 4 given the larger dose of glycogen, 3 survived the toxic dose of serum. Glycogen is rapidly converted into simpler sugars in the circulation and therefore in itself could not have had the effect observed, but this does not exclude the possibility that colloidal disturbance produced by its injection can persist for some time after the disappearance from the circulation of both the glycogen and its products. The animals weighed 335 gm. ± 35 gm. Four controls, weighing respectively 345, 325, 340 and 365 gm. were killed typically by 0.4 c c. of horse serum, and two survived 0.3 c c. The results appear to indicate that only after 24 hours is there inhibition by the larger dose of glycogen. This interpretation is probably fallacious, as will be pointed out later, and no real inhibitory action can be ascribed to glycogen. The toxic dose was controlled on the second day.

Witte Peptone.—This is a protein colloid, which is rapidly fatal in a dose of 0.94 mg. per gram animal weight, with symptoms and anatomic findings practically identical with those of anaphylactic shock. In this series we employed doses of 0.005 and 0.010 mg. per gram animal weight in 0.1% and 0.2% solutions, which were followed by slight reactions in all the animals. They were treated 21 days after sensitization. The toxic dose of horse serum, 0.2 c c., was rapidly fatal for all 4 animals used as controls. Of 3 animals given the smaller dose of peptone and then horse serum one-half hour later, 2 were severely shocked and recovered, the other died typically in 4 minutes. Of the 3 given larger doses of peptone, 2 were only slightly shocked by the subsequent dose of horse serum and 1 died typically in 19 minutes. Twenty-four hours after the peptone, 2 of the 3 given smaller doses of peptone died typically on injection of serum, and the 3rd survived moderate shock. Of 3 given the larger dose of peptone, 2 died typically and the 3rd survived severe shock. This experiment indicates that Witte peptone protects for a period of at least one-half hour, but that at the end of 24 hours this protective influence has almost entirely disappeared. Further details are regarded as unnecessary in view of the well-known inhibitory action of Witte peptone.

Kephalin.—This is a nonprotein colloid, which is practically harmless. In the first two groups in the present series, we employed doses of 0.014 and 0.028 mg. per gram of animal weight, which were followed by slight reactions in 10 of 17 animals. Considerably larger doses were not fatal. The kephalin was prepared according to Mathews.[3] In order to remove cholesterol and particularly

[3] Physiological Chemistry, 1915, p. 571.

lecithin, the solution in ether and precipitation with alcohol was repeated 5 times. In the first group of animals, used 26 days after sensitization, 0.1 c c. of horse serum was found to be fatal. Five animals were given the smaller dose of kephalin; 3 were moderately shocked by the toxic dose of horse serum given one-half hour subsequently, and 2 died typically. Of 5 given the larger dose of kephalin, all survived the toxic dose of horse serum, and only 2 were severely shocked. In the second group, sensitized for 20 days, 7 animals were given the protective dose 24 hours before the toxic dose (in this instance 0.3 c c.) of horse serum, and 5 died typically, the others exhibiting only moderate shock. From other subsequent experiments it was found that inhibition was somewhat reduced after one hour. Thus, the apparent inhibition by kephalin is high at one-half hour, reduced at one hour and practically absent at 24 hours. It must be admitted that the one-half hour experiments probably exaggerate the appearance of protection, since the toxic dose, although controlled, is small, and may represent a dose not necessarily fatal for a large group of animals. Nevertheless, the results were sufficiently encouraging to justify further experiment.

Subsequent experiments were conducted with Armour kephalin in 0.5% solution, used in doses roughly ranging between 0.02 and 0.1 mg. per gram of animal weight. Controls were practically unaffected by these doses. The first group of experiments was set up with the idea of determining the protective influence of kephalin in increasing doses against a common toxic dose of serum and also to determine roughly the protection against increasing doses of serum. Varying the dose of kephalin had no regular influence on protection. The largest dose of kephalin (5.0 c c. of 0.5% suspension), however, protected against almost three times the dose of horse serum known to be fatal, a result that is particularly interesting when, as will be shown later, this dose does not indicate maximal inhibition. Large doses of salt solution were effective, but in the small number tried, not so much so as is kephalin.

An additional set of experiments was arranged to determine the protective influence of increasing doses of kephalin against a common fatal dose of serum. The largest doses of kephalin are less effective than smaller doses. The toxic doses were given one hour after the kephalin and not at the height of inhibition. This gave probably a more sensitive test of differential dosage of kephalin.

These experiments were inadequate in certain respects, particularly as to conclusive evidence of the greater effectiveness of smaller doses of kephalin and in the number of salt solution controls. Therefore, the following experiments were made. Of 6 animals given 1 c c. of 0.5% kephalin solution, 5 survived a toxic dose of serum, whereas of 6 given 5 c c. of 0.5% kephalin, only 1 survived the toxic dose of serum. This group makes it probable that the smaller doses of kephalin are more inhibitory than larger doses, whereas there is little relative difference between the two comparable doses of salt solution.

In the experiments reported, 54 animals were given kephalin before the toxic dose of horse serum, and of these 26 died of anaphylactic shock.

DISCUSSION

Of the colloids employed, congo red is the one in truly suspensoid form and is further removed from the order of colloidal phenomena probably encountered in anaphylaxis than are the other colloids. It is ineffectual in inhibiting shock. Although sodium chloride is used in the menstruum, there is little doubt that the other colloids are in

emulsoid form. Of these, the Witte peptone is the only protein colloid and gives excellent results. Kephalin is the only lipoid of the group and also gives apparently good results. This is in accord with other work with lipoids. Jobling and Peterson [4] claim that repeated subcutaneous injections of lipoids produces inhibition by increasing the antiferment of the blood. Achard and Flandrin [5] believe that they demonstrate inhibitory action of ovolecithin. Duprez [6] regards this a general property of lipoids and claims to have produced inhibition by the use of the acetone insoluble Wassermann antigen of Bordet. We cannot regard Duprez' experiments as conclusive, since adequate details and controls are not given. It would appear, however, that certain protein colloids and certain lipoids produce conditions which inhibit anaphylactic shock, and since we know that mild shock will also protect against doses which otherwise would produce fatal shock, it would appear possible that true desensitization is of the order of colloidal phenomena produced by injection of these protein colloids and lipoids. That these phenomena are identical with those produced in anaphylactic shock is not probable, as indicated by the studies of Hanzlik and Karsner.[2]

SERIES B.

There then arises the question as to whether or not the method gives results that are sufficiently conclusive. Many of the reports on desensitization and inhibition have been based on the same method, and many investigators consider that the variation in sensitiveness to the homologous protein is sufficiently small to permit of conclusions. Unless this assumption be correct, the determination of relative degrees of protection is highly fallacious, for it is a practical impossibility so to extend the number of experiments as to avoid the error of random sampling. In order to test this assumption, an extensive series of animals was studied in order to determine the variations in sensitiveness.

For this purpose, guinea-pigs were sensitized by subcutaneous injection of 0.05 c c. of horse serum and were tested 21 days subsequently. It was found that 0.3 c c. serum intravenously was fatal for 2 of 3 animals. Nevertheless, of 8 animals given 0.6 c c., 3 survived. In a similar series tested 22 days after sensitization, 4 animals given 0.1 c c. died of typical anaphylactic shock, but of 6 given 0.3 c c., 3 recovered. In the latter group, 0.1 c c., fatal for 4 animals in succession, might easily have been regarded as a fatal dose. In order to test the method of sensitization, a group of animals was given 0.05 c c. of horse serum subcutaneously on each of 3 successive days, and the toxic dose was given

[4] Jour. Exper. Med., 1914, 20, p. 468.
[5] Compt. rend. Soc. de biol., 1911, 71, p. 91.
[6] Ibid., 1922, 86, p. 285.

21 days after the last sensitizing dose. This method failed to produce uniform sensitization, since 1 of 2 given 0.4 c c. died typically, and 2 of 3 given 0.6 c c. died typically. Two animals were given 0.5 c c. undiluted serum, and 1 died typically.

Since kephalin, by the earlier method, appeared to give promising results, a group of animals was used in which 0.3 c c. of horse serum was found to produce death 21 days after sensitization. Eight animals were given 0.6 c c. of horse serum, and 5 died typically. Eight animals were given 2 c c. of 0.5% kephalin (Armour) suspension one-half hour before being given 0.6 c c. of horse serum, and 6 died typically. Eight were given 2 c c. of 0.85% NaCl solution one-half hour before being given 0.6 c c. of horse serum, and 7 died typically. Since the controls and the salt solution controls died in essentially the same proportion as the kephalin animals, no real protection can be postulated. Even admitting that a necessarily fatal dose was not established, the number of deaths in the kephalin animals is so great that further experiment with this substance does not seem justified.

DISCUSSION

The results indicate that within its practical limitations, the method is qualitative rather than quantitative. Although the earlier study with heterologous serums and those with the various colloids are suggestive, it must be recognized that a strict interpretation on a qualitative basis leaves the problem open. The same criticism may be directed against reports such as those of Duprez,[6] of Besredka,[7] of Moldovan and Zolog,[8] and others. Hussey apparently recognizes the limitations of the method, and his results are based on protection of an entire group of animals. Experiments on the basis of this method can only be regarded as indicative of inhibition when all animals subjected to the toxic dose survive. The results would be strongly suggestive if a considerable proportion of a reasonably large number of animals survived.

CONCLUSIONS

The experiments clearly indicate that the suspensoid colloid Congo red does not inhibit anaphylactic shock.

Although not definitely proved in these experiments, there is no doubt that the protein colloid Witte peptone is definitely inhibitory to anaphylactic shock.

Inhibition of anaphylactic shock by lipoid colloids such as kephalin is suggested by experiments in the hands of others and by the present study, but a critical examination of the method does not conclusively support this hypothesis.

[7] Ann. d. l'Inst. Pasteur, 1923, 37, p. 935.

[8] Compt. rend. Soc. de biol., 1923, 39, p. 1239; Moldovan, J., and Zolog, M.: Ibid., p. 1242.

There is no ground at present for assuming that colloids as such inhibit anaphylactic shock, but this cannot as yet be applied to the hypothesis that anaphylactic shock is due to disturbance of colloidal balance.

Experimental anaphylactic shock produced in sensitized guinea-pigs by intravenous injection of homologous serum permits no satisfactory interpretation of relative degrees of sensitization or inhibition, either on the basis of clinical symptoms or fatal results.

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